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13. Abstract *(Maximum 200 Words)*

The purpose of this project is to investigate whether or not regulation of expression of the BRCA-1 gene in breast epithelial cells exposed to polycyclic aromatic hydrocarbons (PAHs) is mediated by the aryl hydrocarbon receptor (AhR). *The scope* of the project is to examine whether or not the AhR complexed with the AhR-nuclear transporter (ARNT) protein, binds to several xenobiotic responsive elements (XRE) strategically located at –539 bp (CCGTGGAA=Cyp1A1-like) and +20base pairs (bp) (GCGTG=XRE-1) from the transcription start site on exon-1A. Two additional XREs (GCGTG) have been localized at –107 bp in the intervening sequence upstream (XRE-2) and +218 bp (XRE-3) into exon-1B. Findings of the experiments conducted in year 2 were: 1) Completed testing of deletion contructs for CYP1A1, XRE1, XRE2, and their combinations. 2) Confirmed in binding experiments that the candidate BRCA-1 XREs are targets by proteins contained in nuclear extracts obtained from MCF-7 cells. 3) Obtained preliminary evidence by electromobility shift assay that the BRCA-1 XREs are targeted by nuclear protein complexes containing the aromatic hydrocarbon receptor. 3) Investigated the time-dependent effects of B[a]P on binding of the AhR to candidate BRCA-1 XREs. These preliminary data suggest that the activated AhR may target XREs harbored in the flanking region of the BRCA-1 gene, thus regulating its expression.

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Introduction

The subject of the research is to investigate whether or not exposure to polycyclic aromatic hydrocarbons (PAHs) may be a risk factor in the onset of mammary neoplasia by altering transcription of the tumor suppressor gene, BRCA-1. The *purpose* of this project is to investigate whether or not changes in the expression of the BRCA-1 gene in breast epithelial cells induced by PAHs is mediated by the aryl hydrocarbon receptor (AhR). *The scope* of the project is to examine whether or not the AhR complexed with the AhR-nuclear transporter (ARNT) protein, binds to several xenobiotic responsive elements (XRE) strategically located at –539 bp (CCGTGGAA=Cyp1A1-like) and +20base pairs (bp) (GCGTG=XRE-1) from the transcription start site on exon-1A. Two additional XREs (GCGTG) have been localized at –107 bp in the intervening sequence upstream (XRE-2) and +218 bp (XRE-3) into exon-1B.

Body

Synopsis

The tasks of year 2 of the project focused on 1) completing the testing of deletion contructs for CYP1A1, XRE1, XRE2, and their combinations; 2) Confirming in binding experiments that the candidate BRCA-1-XREs are targets by proteins contained in nuclear extracts obtained from MCF-7 cells; 3) Obtaining preliminary evidence by electromobility shift assay that the BRCA-1-XREs are targeted by the aromatic hydrocarbon receptor; and 4) Investigating the time-dependent effects of B[a]P on binding of the AhR to candidate BRCA-1-XREs. The overall goal of these objectives was that of investigating the contribution of the proposed binding sites for AhR-ligands to regulation of the BRCA-1 promoter. The sites of interest were a CYP1A1-like element (TCCGTGAGAA) homologous to an AhR-responsive domain found in the CYP1A1 gene and three consensus xenobiotic responsive elements (XRE) (GCGTG=XRE-1, XRE-2, XRE-3) spatially arranged upstream of the transcription start sites of exon-1A and exon-1B.

Deletion constructs

Deletion of the CYP1A1-like and XRE-1 elements reduced (1.6- and 2.0-fold respectively) basal (Figure 1) and estrogen-dependent (Figure 2) transcription of the BRCA-1 promoter, which in contrast was increased by 3.0-fold upon deletion of XRE-2. These data contributed to clarifying the contribution of the candidate XREs to regulation of the BRCA-1 gene

We have previously reported (please see 2001 Annual Progress Report) that mutation of the CYP1A1-like element reduced by 2.0-fold the basal reporter activity of the BRCA-1 promoter in MCF-7 cells cultured in control DMEM medium (Fig. 3B of previous report). Thus data from both mutational as well deletion (current report) analyses support the notion that the XRE contained in the CYP1A1-like element may be required for basal activity of the BRCA-1 promoter. In contrast to what seen with mutation of XRE-1 (+ 2.0-fold), deletion of this element resulted in a 2.0-fold reduction of basal BRCA-1 transcription. A possibility is that deletion of the GCGTG domain may have altered the spatial arrangement of the BRCA-1 promoter independent of the effects of the XRE-1. Nevertheless, deletion of XRE-2 confirmed the data obtained with mutational analysis in that removal of the XRE-2 produced a nearly 3.0-fold increase in basal and estrogen-dependent activity of the BRCA-1 promoter. Our overall interpretation of these data is that these XREs contribute to both positive (XRE-1) and negative (XRE-2) regulation of the BRCA-1 gene, possibly by modulating the participation of an array of yet undefined transcription factors.

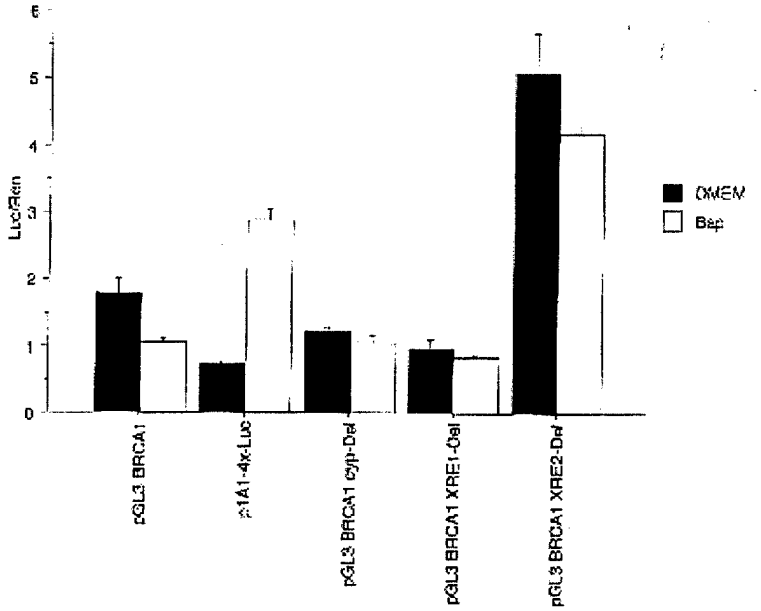

Figure 1. Effects of deletion of XRE-1 and XRE-2 on transcriptional activity of the BRCA-1 promoter. Measurements are relative luciferase reporter units.

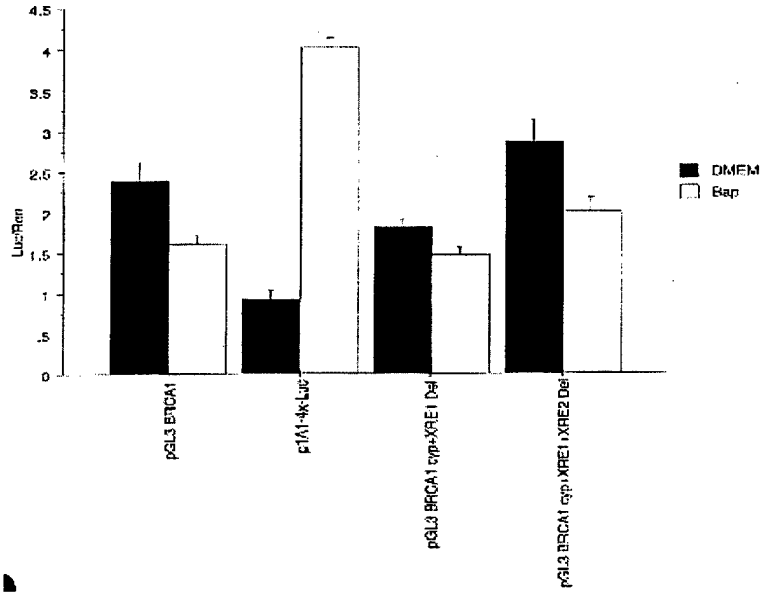

Figure 2. Effects of combinatorial deletions of XREs on transcriptional activity of the BRCA-1 promoter. Measurements are relative luciferase reporter units.

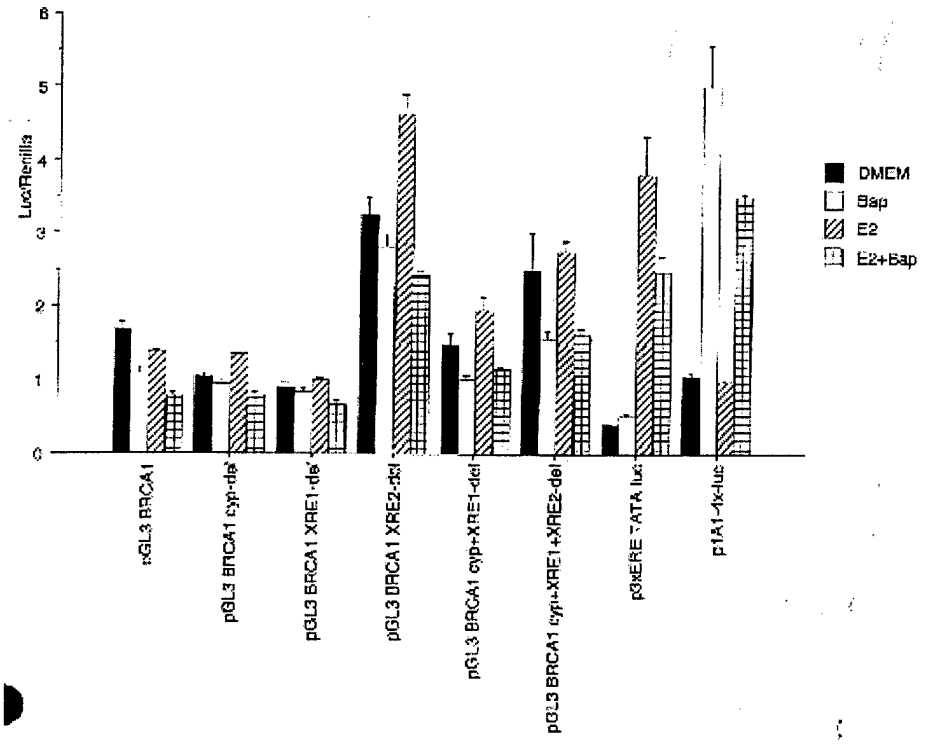

Figure 3. Effects of deletions of XREs on transcriptional activity of the BRCA-1 promoter under conditions of estrogen (E2) stimulation. Measurements are relative luciferase reporter units.

Binding of nuclear proteins to candidate XREs and contribution of the aromatic hydrocarbon receptor.

To clarify the contribution of the AhR to regulation of the BRCA-1 promoter, we prepared nuclear extracts from MCF-7 cells treated with B[a]P for various periods of time. Nuclear extracts were incubated with oligonucleotides harboring the XRE of interest. The results of preliminary experiments indicate that binding to a positive control oligonucleotide containing a consensus sequence for a dioxin-responsive element (DRE) increased in a time-dependent fashion upon treatment with B[a]P. The addition of an antibody against the AhR produced a supershifted band confirming the participation of the AhR at the DRE (data not shown). Similar results were obtained with oligonucleotides containing the CYP1A1-like (Figure 4), the XRE-1 and XRE-2 (data not shown). The data presented in Figure 4 are representative for CYP1A1, XRE-1 and XRE-2. Experiments addressing the ability of the AhR to target XRE-3 are currently in progress. These data indicated that binding to the target oligonucleotide was increased at

3 h after treatment with B[a]P and declined thereafter. Binding was washout in the presence of 100-fold excess of cold (unlabeled) nucleotide but not by the addition of 100-fold excess non-specific oligonucleotide. Co-incubation of the nuclear extracts with the CYP1A1-containing XRE plus a specific antibody against the AhR produced a supershift (higher molecular weight band) representing the oligonucleotide-AhR heterocomplex. These data show for the first time that binding of the AhR at the XREs may represent a mechanism for direct regulation by AhR-ligands of BRCA-1 transcription. The contribution of ancillary proteins (ARNT) to formation of this hetero-complex is currently under investigation. The potential implications of these findings are that regulation of BRCA-1 transcription by the activated AhR may alter the regulation of BRCA-1 and influence its participation in DNA repair, thus influencing the propagation of DNA damage. We are currently performing mutational electromobility shift analysis to confirm that the candidate XREs are bona-fide binding sites for the AhR.

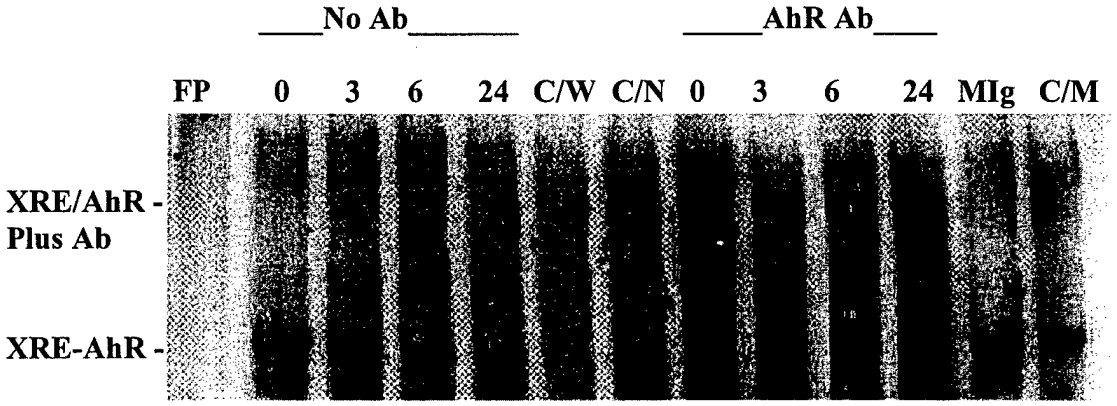

Figure 4. Electromobility shift analysis of AhR binding to cancidate XREs in the BRCA-1 promoter. Bands represent binding by nuclear proteins to the CYP1A1-XRE oligonucleotide. FP= free probe; 0, 3, and 6 are h of incubation in the absence or presence of antibodies against the AhR; C/W= 100x Cold wild-type; C/N= 100x cold non-specific; Mig= mouse IgG; and C-M mutant CYP1A1-XRE.

Key research Accomplishments

- Completed testing of deletion contructs for CYP1A1, XRE1, XRE2, and their combinations.
- Confirmed in binding experiments that the candidate BRCA-1 XREs are potential targets by proteins contained in nuclear extracts obtained from MCF-7 cells expose to benzo[a]pyrene.
- Obtained preliminary evidence by electromobility shift assay that the BRCA-1 XREs are targeted by nuclear protein complexes containing the aromatic hydrocarbon receptor.
- Investigated the time-dependent effects of B[a]P on binding of the AhR to candidate BRCA-1 XREs.

Reportable Outcomes

1. Brandon D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo. Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[*a*]pyrene to 7*r*,8*t*-Dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Cancer Res 2002 62: 113-121.

2. Brandon D. Jeffy, Ryan B. Chirnomas, and Donato F. Romagnolo. Epigenetics of breast cancer: polycyclic aromatic hydrocarbons as risk factors. Environ. Mol. Mutagenesis, Vol 39, 2-3, 2002.

3. The activated aromatic hydrocarbon receptor regulates basal and estrogen-dependent activity of the BRCA-1 promoter. Jeffy, Ryan B. Brandon D. Jeffy, and Donato F. Romagnolo. Manuscript in submission (Cancer Research).

The support of the US Army Medical Research and Materiel Command has been acknowledged in the Acknowledgment section of these manuscripts:

1. Abstracts presented at the Meetings of the Breast Cancer and Environmental Mutagens Conference, Environmental Mutagen Society, Research Triangle Park, NC, September 22-25, 2001.
2. Posters presented at the Arizona Cancer and Southwest Environmental Health Sciences Centers, The University of Arizona, Tucson, AZ
3. The information being gathered through the execution of the experiments supported by this award is being used as the backbone of a Ph.D. project for Brandon Jeffy, who is currently working on this project in the laboratory of the P.I. Mr. Jeffy is a Ph.D. candidate in the Cancer Biology Interdisciplinary Program at the University of Arizona, Tucson, AZ.

Conclusions

Summary

Based on the data obtained through the completion of the experiments outlined in the Body section of this report, we can conclude that exposure to ligands of the aromatic hydrocarbon receptors regulates transcription of the BRCA-1 gene, likely through XREs comprised in the BRCAì1 promoter. The mechanism being proposed is that through binding to AhR-binding domains, the AhR regulates the expression of BRCA-1. As previously suggested at the end of year 1, the candidate responsive elements (CYP1A1 and XREs) appear to have distinct functions. The CYP1A1 appears to be necessary for constitutive expression of BRCA-1 and may represent a mechanism for maintenance of BRCA-1 expression, possibly in response to short-term/low-dose exposure. In contrast, the XRE-2 may be a negative regulator of basal and estrogen-regulated BRCA-1 transcription. The functionality of these sites as AhR-binding elements has been tested by binding and electro-mobility shift assays proposed in Specific Aim#2. These experiments have provided preliminary but important evidence the candidate XREs may be target for binding by the AhR.

Importance and Implications

The findings of this Second Annual Report confirm the original assumption that AhR-ligands may contribute to basal and estrogen regulation of BRCA-1 expression. These conclusions have been confirmed with the results of the transfections with deletion constructs. Experiments conducted in year 2 have clarified that the AhR may contribute to formation of a transcription complex at the XREs. The involvement of ancillary proteins (ARNT) is currently under investigation and will assist in claryfing the contribution of AhR ligands to regulation of BRCA-1.

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1. Romagnolo, D., Annab, L.A., Lyon, T.T., Risinger, J.I., Terry, L.A., Barrett, J.C., and Afshari, C.A. Estrogen upregulation of expression of BRCA-1 with no effect on localization. Mol. Carcinogen., *22*, 102-109, 1998.
2. Jeffy, B.D., Schultz, E.U., Selmin, O., Gudas, J.M., Bowden, G.T., and Romagnolo, D. Inhibition of BRCA-1 expression by benzo[a]pyrene and its diol epoxide. Mol. Carcinogen., *26*, 100-118, 1999.
3. Jeffy, B.D., Chen, E.J., Gudas, J.M., and Romagnolo, D.F. Disruption of cell cycle kinetics by benzo[a]pyrene: Inverse expression patterns of BRCA-1 and p53 in MCF-7 cells arrested in S and G2. Neoplasia, *2*, 460-470, 2000.
4. Brandon D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo. Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[*a*]pyrene to 7*r*,8*t*-Dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Cancer Res 2002 62: 113-121.
5. Brandon D. Jeffy, Ryan B. Chirnomas, and Donato F. Romagnolo. Epigenetics of breast cancer: polycyclic aromatic hydrocarbons as risk factors. Environ. Mol. Mutagenesis, Vol 39, 2-3, 2002.

Appendices

1. Brandon D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo. Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[*a*]pyrene to 7*r*,8*t*-Dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Cancer Res 2002 62: 113-121.
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[CANCER RESEARCH 62, 113–121, January 1, 2002]

Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[*a*]pyrene to 7*r*,8*t*-Dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene[1]

Brandon D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo[2]

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ABSTRACT

Reduction of BRCA-1 expression through nonmutational events may be a predisposing event in the onset of sporadic breast cancer. In this study, we investigated the mechanisms through which the environmental carcinogen benzo[*a*]pyrene (B[*a*]P) lowered BRCA-1 mRNA levels in breast cancer MCF-7 cells. We report that B[*a*]P does not compromise the stability of BRCA-1 mRNA, but represses transcriptional activity of a 1.69-kb BRCA-1 (pGL3-BRCA-1) promoter fragment that contains both exon-1A and exon-1B transcription start sites. The loss of BRCA-1 promoter activity was accompanied by accumulation of CYP1A1 and BAX-α mRNA and p53 and p21 protein, whereas levels of Bcl-2 mRNA were reduced. The aromatic hydrocarbon receptor ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is not metabolized, did not affect BRCA-1 promoter activity or the cellular levels of BRCA-1 and p53 protein, but it did induce a CYP1A1-like promoter. Conversely, treatment with the B[*a*]P metabolite 7*r*,8*t*-dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo-[*a*]pyrene (BPDE) repressed BRCA-1 promoter activity and protein, while increasing p53 and p21 protein levels. Transient expression of dominant-negative p53 (^{175}Arg→His) counteracted the detrimental effects of BPDE on BRCA-1 promoter activity and protein levels. Similarly, treatment with B[*a*]P, TCDD, or BPDE failed to repress transcription from the pGL3-BRCA-1 construct transfected into ZR75.1 breast cancer cells containing mutated p53 (^{152}Pro→Leu). We conclude that activation of the aromatic hydrocarbon receptor is not sufficient for down-regulation of BRCA-1 transcription, which is, however, inhibited by the B[*a*]P metabolite BPDE through a p53-dependent pathway.

INTRODUCTION

The characterization of many germ-line mutations in familial breast and/or ovarian cancers has confirmed the role of *BRCA-1* as a tumor suppressor gene (1–3). Nevertheless, only a small fraction of sporadic ovarian tumors (4) and no sporadic breast cancers (5) have been shown to harbor mutations in the *BRCA-1* gene. These observations are indicative that alternative mechanisms other than coding mutations need to be considered for *BRCA-1*-mediated oncogenesis (6).

Loss of BRCA-1 expression may result from exposure to DNA-damaging agents (7) and methylation at 5′CpG islands in the *BRCA-1* gene (6, 8, 9). We have directed our attention toward investigating the role of environmental xenobiotics such as PAHs[3] as epigenetic disruptors of BRCA-1 expression. PAHs are classic DNA-damaging and tumor-promoting agents found in industrial pollution, auto exhaust, tobacco smoke, and coal tar (10). Exposure to PAHs elicits a number of genotoxic responses, including mammary tumors in rodents (11), oxidative damage (12), DNA adduct formation, (13–15), and base substitutions (16–18). We (19, 20) and others (21) have documented that estrogen increased cellular levels of BRCA-1 mRNA and protein in breast and ovarian cancer cells. In contrast, our group has recently reported that acute and chronic exposure to B[*a*]P, a prototype PAH, lowered both constitutive and estrogen-dependent expression of BRCA-1 in breast and ovarian cancer cells (22). The reduced potential for BRCA-1 expression correlated with S-phase and G_2-M arrest and accumulation of p53, mdm2, and p21. The fact that cotreatment with the AhR antagonist α-naphthoflavone restored normal cell cycle distribution and BRCA-1 expression (23) suggests that the AhR pathway contributes to down-regulation of BRCA-1. In this study, we investigated the mechanisms through which B[*a*]P lowered BRCA-1 mRNA levels in MCF-7 breast cancer cells. We report that B[*a*]P repressed transcription of the BRCA-1 promoter in MCF-7 breast cancer, but not in ZR75.1 cells containing mutated p53. However, in MCF-7 cells, activation of the AhR pathway by B[*a*]P was not sufficient for down-regulation of BRCA-1 expression. Rather, BRCA-1 promoter activity was inhibited by the metabolite BPDE through a p53-dependent pathway.

MATERIALS AND METHODS

Cell Culture and Chemicals. MCF-7 and ZR75.1 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM/F12 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (Hyclone Laboratories, Inc, Logan, UT) as described previously (19). B[*a*]P and actinomycin D were obtained from Sigma Chemical Co.), BPDE and TCDD were obtained from Midwest Research Institute (Kansas City, MO).

Semiquantitative RT-PCR and RNase Protection Assay. For mRNA studies, cells were plated at a density of 2×10^6 cells/100-mm tissue culture dish and maintained in DMEM/F12 plus 10% FCS. Three dishes were assigned to each experimental treatment. Details concerning the experimental conditions for semiquantitative RT-PCR analysis of BRCA-1 and CYP1A1 mRNA are described elsewhere (22). Briefly, total RNA (400 ng) was incubated with random hexamer primers, Moloney murine leukemia virus reverse transcriptase, RNase inhibitor (Life Technologies, Inc., Gaithersburg, MD), and reverse transcription buffer (Ambion Inc., Austin, TX) at 42°C for 1 h. cDNAs were amplified using the oligonucleotides summarized in Table 1. The amplification products were of the expected sizes, and their authenticity to the GenBank was confirmed by direct sequencing. Preliminary control experiments (data not shown) were carried out to assure that RT-PCR conditions allowed for linear amplification of PCR products.

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[2] To whom requests for reprints should be addressed, at Laboratory of Mammary Gland Biology, 303 Shantz Building, The University of Arizona, Tucson, AZ 85721. Phone: (520) 626-9108; Fax (520) 621-9446; E-mail: donato@u.arizona.edu.

[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; B[*a*]P, benzo-[*a*]pyrene; AhR, aromatic hydrocarbon receptor; BPDE, 7*r*,8*t*-dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; RT-PCR, reverse transcription-PCR; RLU, relative luciferase unit(s); XRE, xenobiotic responsive element.

Table 1 *Primers for RT-PCR*

Gene	Primer	Primer sequence	Size (bp)	Accession number
BRCA-1	DR6	5′-AGCTCGCTGAGACTTCCT GGA-3′	712	U1460
	MDR6	5′-CAATTCAATGTAGACAGACGT-3′		
CYP1A1	A1AF	5′-TAACATCGTCTTGGACCTCTTTG-3′	397	K03191
	A1AR	5′-GTCGATAGCACCATCAGGGGT-3′		
Bax-α	BαF	5′-CTGACATGTTTTCTGACGGC-3′	289	L22473
	BαR	5′-TCAGCCCATCTTCTTCCAGA-3′		
Bcl-2	BC2F	5′-TGCACCTGACGCCCTTCAC-3′	293	M14745
	BC2R	5′-AGACAGCCAGGAGAAATCACAG-3′		

For amplification of the internal standard, 18S rRNA (488-bp), we used the Competimer oligonucleotide module from Ambion. The expression levels of BRCA-1 were quantified by Alpha Imager (Alpha Innotech Inc, San Diego, CA) analysis and corrected for the expression of the control mRNA (BRCA-1/18S). Details of the RNase protection assay for BRCA-1 mRNA are described elsewhere (22). Briefly, a 162-bp BRCA-1 riboprobe encoding a portion of exon 15 was transcribed in the antisense orientation from the transcription vector Triplescript (Ambion). As internal control for RNase protection assay, we transcribed a riboprobe for human cyclophilin from the pTRIcyclophilin vector (Ambion). Relative phosphorimager units for BRCA-1 mRNA were corrected for the expression of the control, cyclophilin mRNA (BRCA-1/cyclophilin).

Western Blotting. Western blotting was performed as described previously (23). Cell extracts were normalized to protein content and separated by 4–12% gradient SDS-PAGE. Immunoblotting was carried out with antibodies raised against BRCA-1 (Ab-2), p53 (Ab-2), and p21 (Ab-1) obtained from Oncogene Research Products (Cambridge, MA). Normalization of Western blots was confirmed by incubating immunoblots with β-actin antibody-1 (Oncogene Research Products). The immunocomplexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

BRCA-1 Promoter Studies. Genomic DNA extracted from ovarian BG-1 cells (American Type Culture Collection) was used for PCR amplification of a 1.69-kb BRCA-1 promoter fragment, using the forward DRPR-F-*Kpn*I (5′-ATCGGTACCGCATTCTGAACCACAGACTCT-3′) and reverse LH9-R-*Bgl*II (5′-ACTAGATCTACCTCATGACCAGCCGACGTT-3′) oligonucleotides. The BRCA-1 primers were designed with *Kpn*I and *Bgl*II linkers, which after restriction digestion generated *Kpn*I- and *Bgl*II-compatible cloning sites. The authenticity to the BRCA-1 sequence deposited in the GenBank (accession no. HSU37574) was confirmed by direct sequencing of the PCR product, which spanned both exon-1A and exon-1B transcription start sites. The BRCA-1 promoter fragment was subcloned into the expression vector pGL3Basic (Promega Corporation, Madison, WI), which was previously digested to create compatible *Kpn*I and *Bgl*II termini, thus generating the pGL3-BRCA-1 luciferase expression construct. For expression studies, the pGL3-BRCA-1 vector was transfected into MCF-7 and ZR75.1 cells by the Lipofectamine-Plus procedure, as described by the manufacturer (Life Technologies). Variations in transfection efficiency were accounted for by cotransfection with plasmids encoding for the *β-galactosidase* or *renilla* gene. Internal standards for luciferase activity were the pGL3Control vector containing an SV40 promoter, and pGL3Basic (empty) (Promega). To control for the efficacy of treatments with B[*a*]P and TCDD, cells were transfected with plasmid p1A1-4X-LUC (a gift from Dr. Pasco, University of Mississippi, University, MS), which contains a CYP1A1 consensus sequence linked to an array of four GCGTG elements. Cell extracts were collected at 24 h after treatment with various concentrations of B[*a*]P, TCDD, or BPDE. Luciferase reporter activity was monitored with a Luminometer 20/20 and expressed as RLU corrected for β-galactosidase (RLU/β-gal) or renilla (RLU/renilla).

RESULTS

Effects of B[*a*]P, BPDE, and TCDD on Expression Profiles. RT-PCR analysis of total RNA from MCF-7 cells revealed that treatment with B[*a*]P and BPDE lowered BRCA-1 mRNA levels (Fig. 1*A*). These changes were accompanied by up-regulation of the *CYP1A1* gene, which encodes for a member of the P450 family of metabolizing enzymes (24). The accumulation of CYP1A1 mRNA confirmed the functionality of the AhR pathway in MCF-7 cells, although B[*a*]P was more effective than BPDE in elevating the content of CYP1A1 transcripts. Treatment with B[*a*]P increased the levels of BAX-α mRNA, whereas transcripts for Bcl-2 were reduced, suggesting that B[*a*]P inversely regulated the expression of Bax-α and Bcl-2. However, neither the Bax-α nor the Bcl-2 mRNA level was affected by BPDE. These distinct expression patterns emphasized the fact that loss of BRCA-1 mRNA in cells treated with B[*a*]P or BPDE did not stem from a general effect on the transcriptional machinery.

Western blot analysis of cell extracts obtained from MCF-7 cells revealed that B[*a*]P lowered BRCA-1 protein levels, while increasing p53 (Fig. 1*B*). Expectedly, treatment with BPDE significantly reduced BRCA-1 protein, whereas the cellular p53 and p21 levels were elevated (Fig. 1*C*). In contrast, treatment with 10 nM TCDD did not alter BRCA-1, p53, or p21 protein levels, but elicited the accumulation of CYP1A1 mRNA levels (data not shown; Ref. 22).

B[*a*]P Does Not Compromise BRCA-1 mRNA Stability but Reduces Promoter Activity. The expression data of Fig. 1 indicated that B[*a*]P and BPDE activated multiple, perhaps overlapping, signal transduction pathways, which must be regarded as an integral part of a cellular network. In this context, we were interested in determining whether inhibition of BRCA-1 expression by B[*a*]P resulted from reduced stability of BRCA-1 mRNA. Data from RT-PCR (Fig. 1*A*) and RNase protection assay (Fig. 2*A*) experiments revealed that, compared with DMEM, the levels of BRCA-1 mRNA corrected for the cyclophilin mRNA were reduced 3.0-fold by treatment of MCF-7 cells with B[*a*]P. To examine the effects of B[*a*]P on BRCA-1 mRNA stability, we compared the rate of decay of BRCA-1 transcripts in

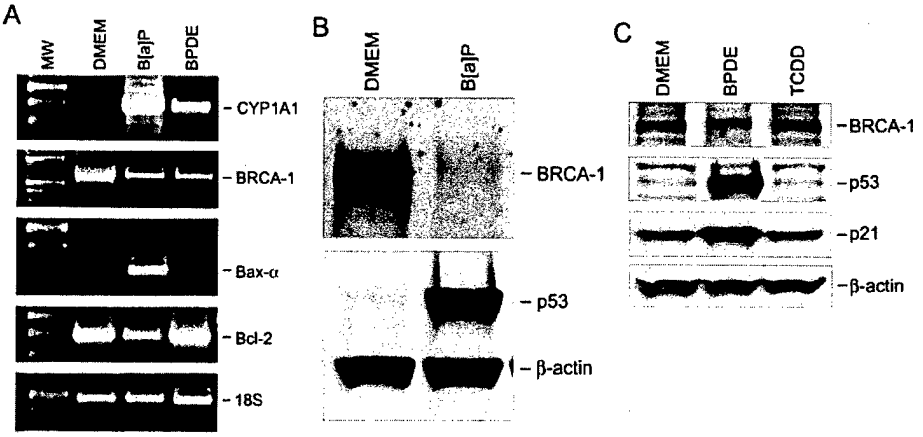

Fig. 1. Effects of B[*a*]P, BPDE, and TCDD on expression profiles. *A*, MCF-7 cells were cultured for 24 h in basal DMEM/F12 plus 10% FCS (*DMEM*), or DMEM plus 5 μM B[*a*]P or 500 nM BPDE. Semiquantitative RT-PCR analysis was performed as described in "Materials and Methods." Bands represent RT-PCR products for CYP1A-1 (397 bp), BRCA-1 (712 bp), Bax-α (289 bp), Bcl-2 (293 bp), and control ribosomal 18S RNA (*18S*; 488 bp) from input cDNA corresponding to 400 ng of total RNA. *MW*, DNA molecular weight markers. *B*, Western blot analysis of BRCA-1 and p53 in cells cultured in DMEM or DMEM plus 5 μM B[*a*]P. *C*, bands are immunocomplexes for BRCA-1, p53, and p21 in cells cultured in DMEM or DMEM plus 500 nM BPDE or 10 nM TCDD. Bands for β-actin are control immunocomplexes.

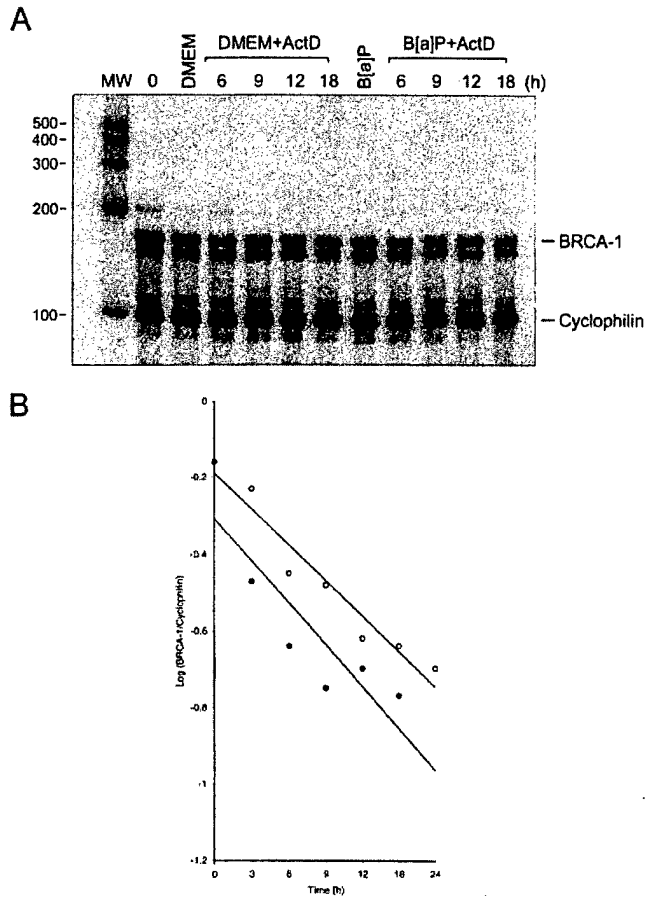

Fig. 2. B[*a*]P does not reduce stability of BRCA-1 mRNA. MCF-7 cells were cultured for 24 h in DMEM/F12 plus 10% FCS (*DMEM*) or DMEM plus 5 μM B[*a*]P. At the end of the incubation period, cells were cultured for various periods of time (6, 9, 12, and 18 h) in the presence or absence of 5 μg/μl actinomycin-D (*ActD*). Cells were harvested, and BRCA-1 mRNA levels were measured by RNase protection assay in 10 μg of total RNA as described in "Materials and Methods." *A*, bands are RNase-protected fragments for BRCA-1 or the internal standard cyclophilin. The doublet is the result of extended digestion of the BRCA-1 mRNA duplex. *MW*, RNA molecular weight standards (bp). *B*, decay of BRCA-1 mRNA in MCF-7 cells cultured in DMEM (○) or DMEM plus B[*a*]P (●). Data are expressed as the semilogarithmic value of BRCA-1/cyclophilin mRNA at each time point.

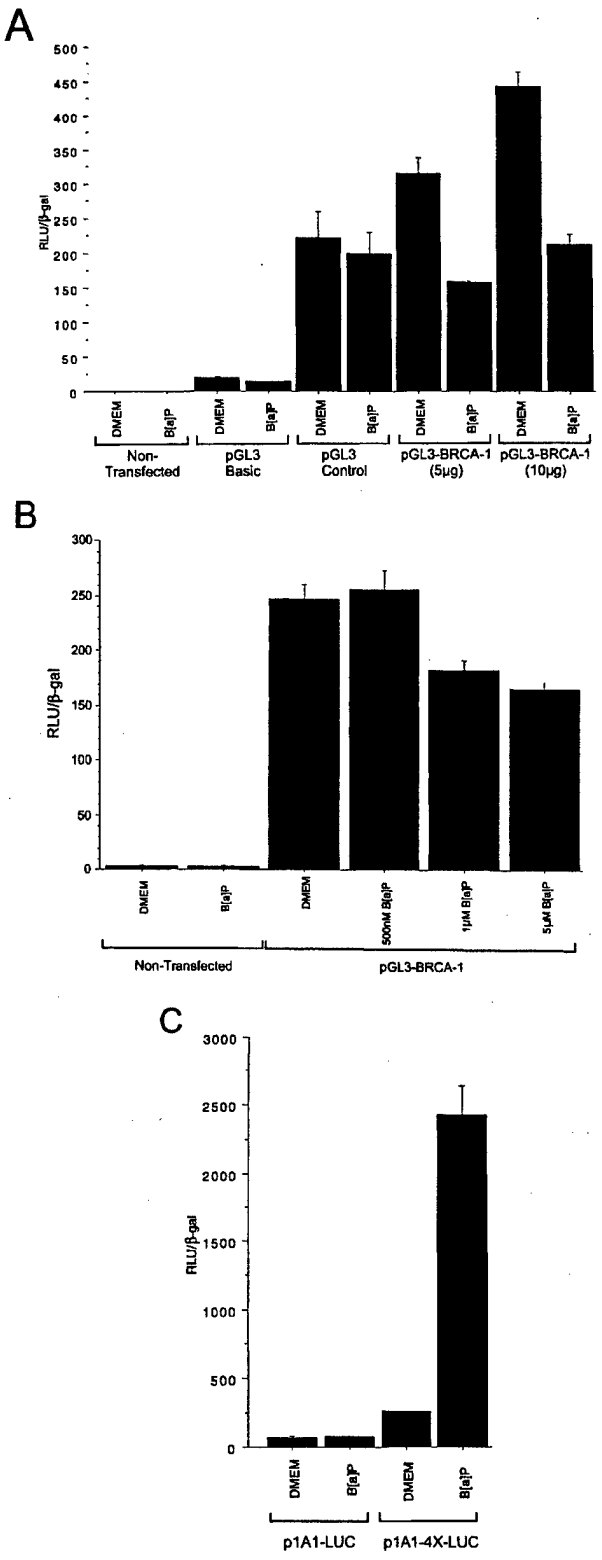

Fig. 3. B[*a*]P inhibits transcription activity of the BRCA-1 promoter. *A*, MCF-7 cells were transiently transfected with the empty pGL3Basic vector or vectors containing a luciferase reporter cassette under the control of the Simian SV40 (*pGL3Control*) or BRCA-1 (*pGL3-BRCA-1*) promoter. RLU were measured after cells were cultured for 24 h in DMEM/F12 plus 10% FCS (*DMEM*) or DMEM plus 5 μM B[*a*]P. *B*, effects of treatment for 24 h with various concentrations of B[*a*]P on RLU in MCF-7 cells transfected with 10 μg of pGL3-BRCA-1. *C*, induction by B[*a*]P (5 μM) of the promoter construct p1A1-4X-LUC (1 μg) containing four XREs. p1A1-LUC is the empty vector lacking the XREs. *Columns* represent mean RLU corrected for β-galactosidase ± SD (*bars*) from two independent experiments performed in triplicate.

control and B[*a*]P-treated cells. After MCF-7 were precultured for 24 h in DMEM/F12 containing 10% FCS with or without B[a]P, culture medium was replaced with fresh DMEM containing 5 μg/ml actinomycin D to inhibit the production of new transcripts in the presence or absence of B[*a*]P. Kinetics of disappearance were assessed by calculating at 3, 6, 9, 12, and 18 h the relative levels of BRCA-1 mRNA corrected for the cyclophilin mRNA. The temporal changes depicted in Fig. 2*B* document that the kinetics of disappearance of the protected BRCA-1 fragment followed a first-order process that was not influenced by B[*a*]P. In fact, the half-life of the BRCA-1 transcript was ~12 h in both control and B[*a*]P-treated cells.

These results suggested that loss of BRCA-1 expression in cells treated with B[*a*]P was likely not a result of increased degradation of BRCA-1 mRNA and prompted further investigations to assess whether B[*a*]P interfered with regulation of transcription at the BRCA-1 promoter. Fig. 3*A* diagrams the luciferase activity detected in MCF-7 cells transfected with pGL3-BRCA-1 in the presence or absence of B[*a*]P. Compared with the RLU measured in cells transfected with the empty pGL3Basic vector, luciferase units corrected for β-galactosidase increased, although not proportionally, 16.0- and 22.0-fold in cells transfected with 5 or 10 μg of pGL3-BRCA-1.

However, after treatment with B[*a*]P, the reporter activity was reduced by 2.2- and 2.0-fold in MCF-7 cells transfected with 5 or 10 μg of the pGL3-BRCA-1 vector, respectively. The RLU detected after transfection with 1 μg of the internal pGL3Control vector were not influenced by treatment with B[*a*]P and were 10.0-fold higher than those measured in cells transfected with the pGL3Basic lacking a promoter element.

In parallel experiments (Fig. 3*B*), we assessed the dose-dependent effects of B[*a*]P in MCF-7 cells transfected with 10 μg of the pGL3-BRCA-1 vector. A B[*a*]P concentration of 0.5 μM did not influence RLU, whereas doses of 1 and 5 μM B[*a*]P significantly reduced luciferase activity by 1.5- and 1.6-fold, respectively. The reporter activity in control cells transfected with the positive control p1A1-4X-LUC was 4.0-fold higher than that produced by the p1A1-LUC vector lacking the four XREs and was increased an additional 10-fold in the presence of B[*a*]P (Fig. 3*C*).

BPDE but not TCDD Represses BRCA1 Promoter Activity. The data shown in Fig. 1*C* illustrated that, at least at the concentration (10 nM) used in this study, TCDD did not lower BRCA-1 protein levels. It should be pointed out that in previous studies (22), increasing the concentration of TCDD from 10 nM to 1000 nM affected neither BRCA-1 mRNA nor protein content in MCF-7 cells, although cell viability was reduced by 50 and 80% with 10 and 1000 nM TCDD, respectively. Because the affinity of TCDD for the AhR is ~100-fold higher than that of B[*a*]P but TCDD is not metabolized (25), we envisioned that activation of the AhR pathway was not sufficient for B[*a*]P-mediated repression of BRCA-1 transcription. Rather, we formulated the hypothesis that products of B[*a*]P bioactivation, possibly BPDE, contributed to down-regulation of BRCA-1. To test this contention, we compared the effects of BPDE (100 and 500 nM) and TCDD (10, 100, and 500 nM) on BRCA-1 promoter activity in cells transiently transfected with the pGL3-BRCA-1 construct. In previous studies, concentrations up to 1.2 μM BPDE were used to investigate repair of DNA damage (26). However, we used lower concentrations, ranging from 100 to 500 nM BPDE, which in our hands have been effective in promoting S-phase arrest and loss of BRCA-1 expression in MCF-7 cells (23). The results shown in Fig. 4*A* indicate that treatment for 24 h with 100 or 500 nM BPDE inhibited by 1.5- and 2.2-fold, respectively, transcription from the BRCA-1 promoter. In contrast, the activity of the BRCA-1 reporter construct was not affected by treatment with TCDD at any of the concentrations tested in this study (Fig. 4*B*). The efficacy of the TCDD treatment was confirmed by evidence that it stimulated a dose-dependent increase in the reporter activity of the positive control, p1A1-4X-LUC (Fig. 4*C*). TCDD concentrations of 10 nM stimulated by 2.0-fold the activity of the 1A1-4X promoter compared with that measured in cells treated with equimolar concentrations of B[*a*]P. This effect was attributed to the higher binding affinity of TCDD for the AhR. A TCDD concentration of 500 nM gave a response comparable to that obtained with 5 μM B[*a*]P. These cumulative data suggested that the AhR is not involved directly in BRCA-1 repression but that the metabolite BPDE, or factors regulated by BPDE, contribute, at least in part, to B[*a*]P-dependent inhibition of BRCA-1 transcription.

Repression of BRCA-1 Promoter Activity by B[*a*]P and BPDE Requires Functional p53. On the basis of our published observation that the AhR antagonist α-naphthoflavone counteracted the S-phase arrest and loss of BRCA-1 expression induced by B[*a*]P while preventing the accumulation of p53 (23), we questioned whether the acquisition of p53 functions in MCF-7 cells treated with B[*a*]P contributed to repression of BRCA-1 promoter activity. To test this hypothesis, we cotransfected MCF-7 cells with a plasmid containing a cassette encoding for p53 mutated at position 175 (Arg to His) under the control of the cytomegalovirus promoter (pCVM53mut) subcloned into pCMV (plasmids were gifts from Dr. Bert Vogelstein, The Johns Hopkins University School of Medicine, Baltimore, MD and made available by Dr. J. Martinez, The University of Arizona, Tucson, AZ). The cotransfection of the empty pCMV (data not shown) or

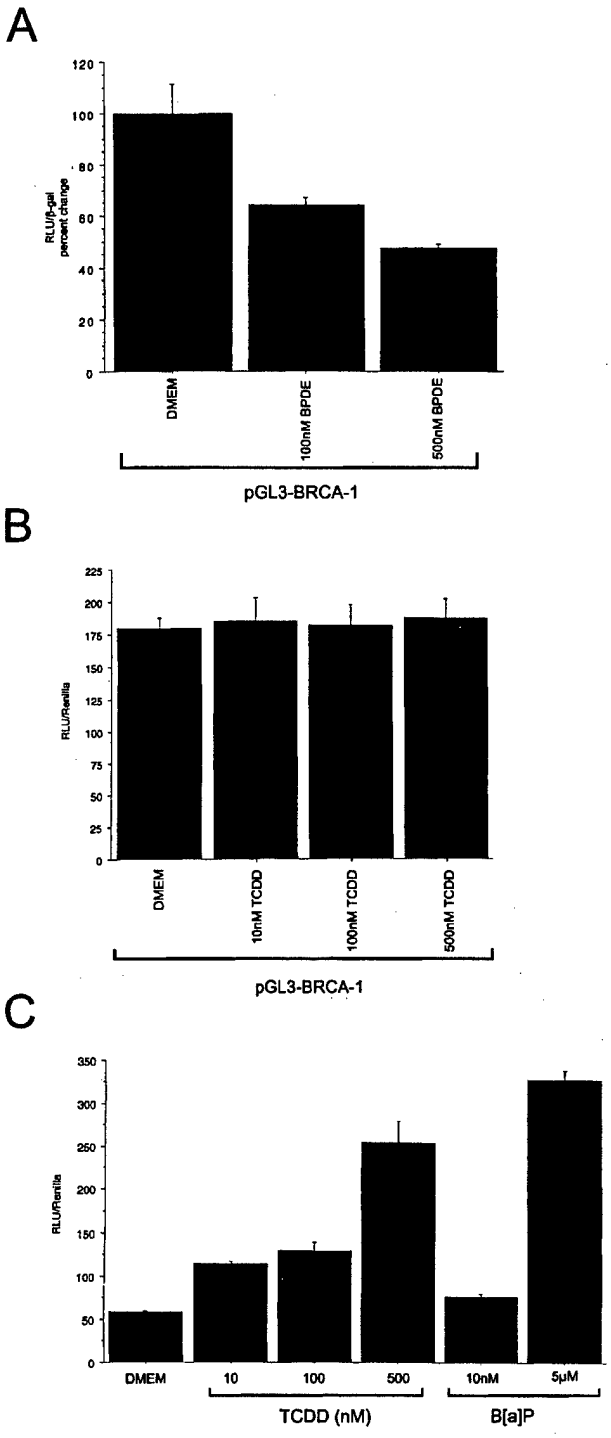

Fig. 4. BPDE, but not TCDD, inhibits BRCA-1 promoter activity. *A* and *B*, MCF-7 cells were transiently transfected with pGL3-BRCA-1 (10 μg). Cells were cultured for 24 h in DMEM/F12 plus 10% FCS (*DMEM*) or DMEM plus 100 nM or 500 nM BPDE (*A*) or 10, 100, or 500 nM TCDD (*B*). *C*, MCF-7 cells were transiently transfected with p1A1-4X-LUC (1 μg) and cultured in DMEM plus various amounts of TCDD (10, 100, and 500 nM) and B[*a*]P (10 nM and 5 μM). *Columns* represent mean RLU corrected for β-galactosidase or renilla ± SD (*bars*) from two independent experiments performed in triplicate.

pCMV53mut vectors with pGL3-BRCA-1 did not influence BRCA-1 luciferase reporter activity in cells cultured in control medium (DMEM; Fig. 5*A*). In contrast, the concomitant transfection of pGL3-BRCA-1 with pCMV53mut, which encodes mutant p53, prevented the loss of BRCA-1 promoter activity (2.0-fold) induced by B[*a*]P. Positive evidence that the pCMV53mut construct expressed p53 was obtained by Western blot analysis (Fig. 5*B*). In control medium (DMEM), p53 levels were low in nontransfected cells or cells transfected with the empty pCMV vector, whereas p53 increased significantly in the presence of B[*a*]P. Conversely, accumulation of p53 was observed in cells cultured in DMEM after transfection with the pCMV53mut vector. The intensity of the p53 immunocomplex increased further after treatment with B[*a*]P, presumably because of coincident immunodetection of endogenous and recombinantly expressed p53.

We next examined the effects of BPDE on activity of the BRCA-1 reporter construct (Fig. 6*A*). The RLU detected in cells transfected with pGL3-BRCA-1 were reduced 1.8-fold by BPDE. In contrast, cotransfection with pCMV53mut restored luciferase activity to control levels. Similar results were obtained after cotransfection of a vector encoding for the human papilloma virus E6 protein, which prevented the loss of reporter activity elicited by BPDE (data not shown). Treatment with BPDE reduced BRCA-1 protein, whereas p53 and p21 levels were increased in cells transfected with the empty pCMV vector (Fig. 6*B*). However, in cells transfected with

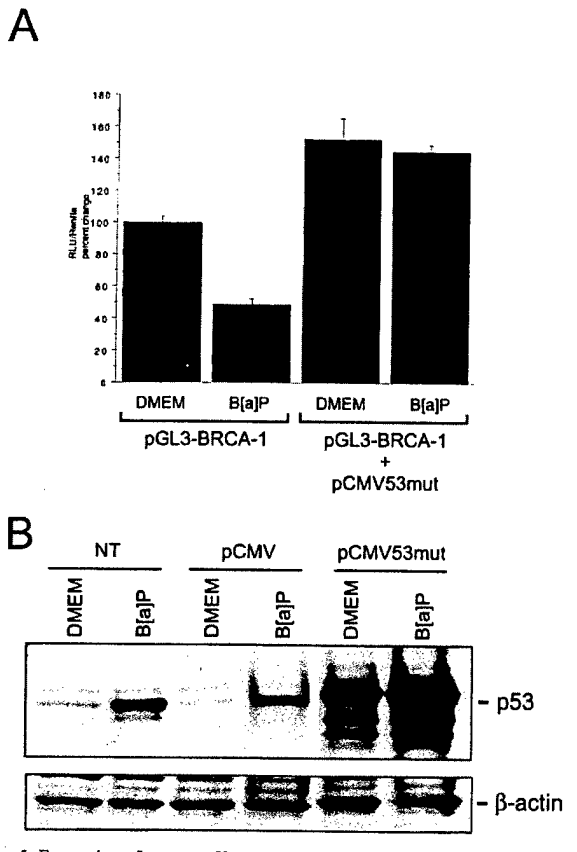

Fig. 5. Expression of mutant p53 counteracts B[*a*]P-mediated loss of BRCA-1 promoter activity. *A*, MCF-7 cells were transiently transfected with pGL3-BRCA-1 (10 μg) or pCMV53mut (3 μg). Cells were cultured for 24 h in DMEM/F12 plus 10% FCS (*DMEM*) or DMEM plus 5 μM B[*a*]P. *Columns* represent mean RLU corrected for renilla ± SD (*bars*) from two independent experiments performed in triplicate. *B*, Western blot analysis of nontransfected cells (*NT*) and cells transfected with the empty vector pCMV (3 μg) or pCMV53mut (3 μg), and cultured for 24 h in DMEM or DMEM plus 5 μM B[*a*]P. *Bands* are immunocomplexes for p53 and control β-actin.

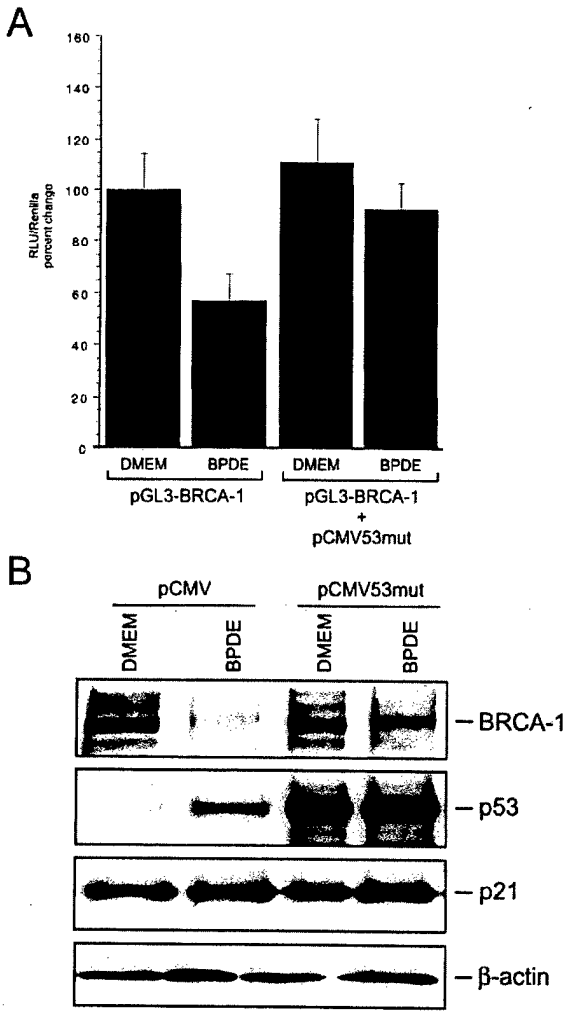

Fig. 6. Expression of mutant p53 counteracts BPDE-mediated loss of BRCA-1 promoter activity. *A*, MCF-7 cells were transiently transfected with pGL3-BRCA-1 (10 μg) or pCMV53mut (3 μg). Cells were cultured for 24 h in DMEM/F12 plus 10% FCS (*DMEM*) or DMEM plus 500 nM BPDE. *Columns* represent mean RLU corrected for renilla ± SD (*bars*) from two independent experiments performed in triplicate. *B*, Western blot analysis of cells transfected with the empty vector pCMV (3 μg) or pCMV53mut (3 μg) and cultured for 24 h in DMEM or DMEM plus 500 nM BPDE. *Bands* are immunocomplexes for BRCA-1, p53, p21, and control β-actin.

pCMV53mut, we detected constitutive expression of p53, whose levels were increased further by BPDE. More importantly, BRCA-1 protein was restored almost to control levels in cells expressing p53mut and treated with BPDE. The cellular content of p21 was elevated by BPDE in cells transfected with pCMV or pCMV53mut, but it was not altered by expression of exogenous mutant p53 in cells cultured in DMEM.

To further test the hypothesis that the metabolite BPDE inhibits BRCA-1 transcription via an effect that is mediated by p53, we examined the regulation on BRCA-1 transcriptional activity in ZR75.1 breast cancer cells, which contain mutated p53 (^{152}Pro→Leu)[4] (27). The functionality of the AhR pathway in ZR75.1 cells was confirmed by evidence of transcriptional activation of the positive control, p1A1-4X-LUC construct, in the presence of 5 μM B[*a*]P (7.0-fold) and 500 nM TCDD (10.0-fold; Fig. 7*A*). Therefore, it appeared that ZR75.1 cells were more responsive than MCF-7 cells to stimulation with equimolar concentrations of TCDD or B[*a*]P. A

[4] Mohammed A. Khan, Laboratory of Human Carcinogenesis, National Cancer Institute (Bethesda, MD), personal communication.

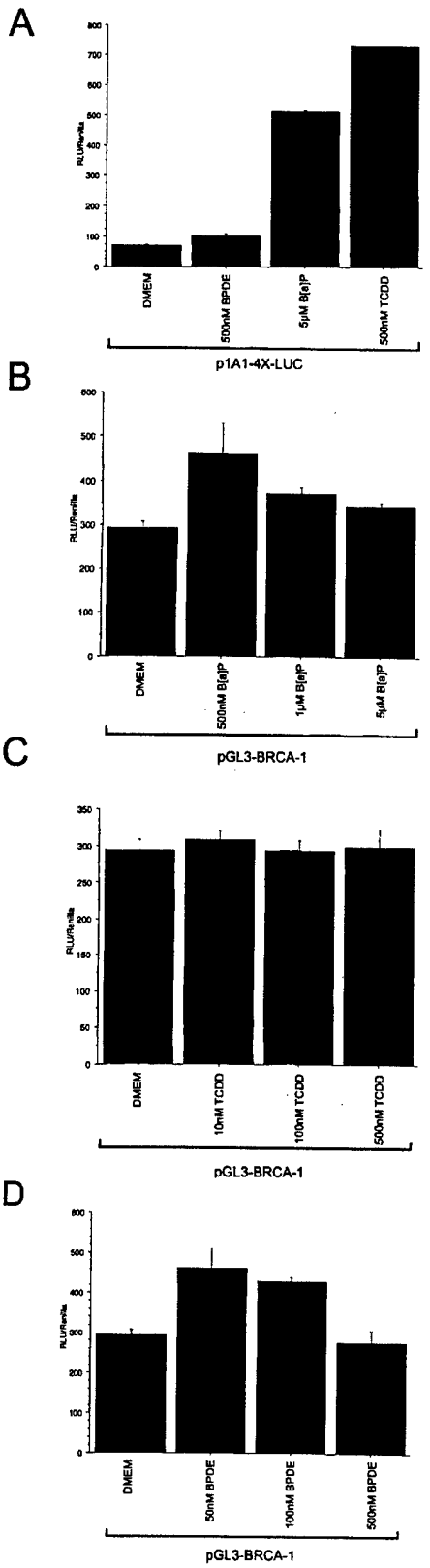

Fig. 7. B[*a*]P and BPDE do not repress BRCA-1 transcription in ZR75.1 breast cancer cells. *A*, ZR-75.1 cells were transiently transfected with p1A1-4X-LUC (1 μg) and cultured in DMEM/F12 plus 10% FCS (*DMEM*) or DMEM plus 500 nM BPDE, 5 μM B[*a*]P, or 500 nM TCDD. *B–D*, cells were transiently transfected with pGL3-BRCA-1 (10 μg) and cultured for 24 h in DMEM or DMEM plus increasing amounts of B[*a*]P (*B*), TCDD (*C*), or BPDE (*D*). *Columns* represent mean RLU corrected for renilla ± SD (*bars*) from two independent experiments performed in triplicate.

slight increase (1.5-fold) in RLU was also observed in ZR75.1 cells transfected with p1A1-4X-LUC and treated with 500 nM BPDE. In contrast, transfection experiments with pGL3-BRCA-1 indicated that TCDD had no effects on BRCA-1 promoter activity (Fig. 7*C*), whereas RLU were increased 1.5-fold in ZR75.1 cells treated with 500 nM B[*a*]P (Fig. 7*B*) or 50 and 100 nM BPDE (Fig. 7*D*). This increase in transcriptional activity of the BRCA-1 promoter was similar to the increase observed in MCF-7 cells cotransfected with dominant-negative p53 (Figs. 5 and 6). Overall, these findings indicated that transcription of BRCA-1 was not repressed by B[*a*]P, BPDE, or TCDD in ZR75.1 breast cancer cells containing mutated p53.

DISCUSSION

The primary objective of this study was to shed some light on the mechanisms responsible for the reduction in BRCA-1 mRNA levels in MCF-7 breast cancer cells exposed to acute levels of B[*a*]P (22, 23). The environmental carcinogen B[*a*]P, a prototype PAH, has been implicated in the development of lung (15) and skin (26) tumors. A generally accepted concept is that the tumor-initiating and -promoting properties of B[*a*]P stem from its metabolic activation by detoxifying enzymes to a pool of end-products, including the highly mutagenic BPDE, which can form DNA adducts (28) and induce transversions at mutational hot spots (18, 29–31). Our working hypothesis is that the reactive metabolite BPDE, selected among many end-products of B[*a*]P metabolism, may alert regulatory cascades that repress BRCA-1 expression. Support for this hypothesis comes from the following: (*a*) No sporadic breast tumors have been shown to harbor mutations in the *BRCA-1* gene (32) but express lower levels of BRCA-1 (33). This implies the existence of epigenetic mechanisms that reduce BRCA-1 expression in the absence of mutational alterations. (*b*) B[*a*]P and BPDE repress constitutive and estrogen-induced expression of BRCA-1 in breast and ovarian cancer cells (22, 23). This effect is not unique to B[*a*]P; other PAHs hamper, in a dose-dependent fashion, BRCA-1 protein levels in MCF-7 cells in the following order: 3-methylcholanthrene > B[*a*]P > benzo[*e*]pyrene.[5] (*c*) Despite their high reactivity, PAH-DNA adducts correlate with low mutation frequencies (2%; Ref. 34), suggesting that physiological rather than genotoxic stresses may be implicated in PAH-dependent tumorigenesis (35). For example, exposure to PAHs contributes to disruption of cell cycle kinetics (19, 36), and PAHs have the ability to circumvent cellular defense mechanisms (18).

In mammalian models, activation of the AhR pathway elicits cell cycle arrest, apoptosis, and expression of genes encoding for enzymes in the cytochrome P450 family, which contribute to bioactivation of AhR ligands (37–39). The AhR is a ligand-activated factor that modulates transcription through interactions with XREs. The XRE-core recognition sequence (5′-GCGTG-3′) is harbored in the 5′ flanking region of several genes, including *CYP1A1*, *CYP1A2*, *UDP-glucuronosyl-transferase*, and the estrogen-inducible *cathepsin-D* (40, 41). The fact that the reporter activity of a PAH-inducible promoter (p1A1-4X-LUC) harboring a tandem of four XREs was greatly induced by B[*a*]P and TCDD provided confirmatory evidence that the AhR pathway was functional in MCF-7 and ZR75.1 cells under present experimental conditions. This notion is also supported by earlier reports documenting regulation by AhR ligands of XRE-containing promoter segments (42). Moreover, we observed that BPDE induces the production of CYP1A1 mRNA in MCF-7 cells and transcription activity of the p1A1-4X-LUC construct in ZR75.1 cells. The latter results deviated from the general model advocating binding of the ligand B[*a*]P to the AhR and the subsequent *trans*-activation of

[5] Our unpublished data.

CYP1A1. We excluded the possibility that the up-regulation of CYP1A1 was attributable to contamination of the BPDE preparation (purity >99%). One possible explanation is that BPDE may induce CYP1A1 via weak binding to the AhR or may be metabolized into a form that is, in turn, capable of binding to or activating the AhR (43). Alternatively, CYP1A1 expression could be induced through AhR-independent pathways (44). Regardless of the mechanism, the fact that accumulation of CYP1A1 mRNA in MCF-7 cells treated with B[*a*]P was accompanied by elevation of Bax-α and loss of Bcl-2 mRNA, whereas in the presence of BPDE the levels of these transcripts were unaltered, lent support to the conclusion that BPDE exerts specific effects on CYP1A1 expression.

With respect to XREs, which are known to confer responsiveness to PAHs, using computer-assisted analysis we have identified an array of candidate XRE consensus sequences in the 1.69-kb BRCA-1 promoter fragment.[6] Although we cannot discard the possibility that binding of the activated AhR to XREs in the *BRCA-1* gene contributed to its negative regulation, the data presented in this report were consistent with a model in which the metabolite BPDE mediated the negative effects of B[*a*]P on BRCA-1 transcription. Because TCDD failed to reduce BRCA-1 promoter activity but did induce transcription from a CYP1A1-like promoter in both MCF-7 and ZR75.1 cells, we concluded that effectors downstream of the AhR, such as BPDE, a product of B[*a*]P bioactivation (45), alerted cellular signals that repressed BRCA-1 promoter activity.

The tumor suppressor gene *p53* encodes for one such effector, whose stability was increased significantly in MCF-7 cells treated with B[*a*]P or BPDE. An increase in p53 in MCF-7 cells exposed to PAH metabolites has been documented in previous reports (36) and was related to DNA adduct formation and delay in S-phase (46). However, more than one pathway has been implicated in the stabilization of p53 in response to DNA damage induced by BPDE, including DNA strand breaks (47) and phosphorylation and poly(ADP) ribosylation of the p53 protein (48). The p53 gene product has been shown to elicit transcription of several genes, including *Bax*, *p21*, and *mdm2* (49), which are involved in cell cycle control and apoptosis (50). On the basis of published observations that expression of p53 and BRCA-1 may be regulated through a feedback loop (51–53), we tested whether inhibition of BRCA-1 transcription by B[*a*]P and BPDE resulted from a gain of p53 functions. In keeping with this concept, transfection of MCF-7 cells containing wild-type p53 (27) with a vector encoding for p53 mutated at amino acid position 175 (Arg to His) abrogated the negative effects of B[*a*]P and BPDE on BRCA-1 promoter activity. Our interpretation of these findings is that transient expression of mutant p53 interfered with normal p53 functions in a *trans*-dominant-negative fashion. Similarly, transient transfection of MCF-7 cells with an expression vector encoding for the E6 human papilloma virus counteracted the negative effects of BPDE on BRCA-1 promoter activity (data not shown). The E6 gene product binds to p53 and leads to suppression of its biological functions (54). From these cumulative data, we concluded that activation of the AhR pathway was required, but not sufficient for B[*a*]P-mediated inhibition of BRCA-1 transcription. Rather, the metabolite BPDE elevated p53, which in turn inhibited BRCA-1 promoter activity. Experiments with ZR75.1 cells corroborated the notion that repression of BRCA-1 transcriptional activity by B[*a*]P or BPDE could not be seen in breast cancer cells containing mutated p53 (^{152}Pro→Leu; Ref. 27). Mutations such as those that occur in the core domain, which consists of amino acids from ~100 to 300, have been shown to result in loss of DNA binding activity of the p53 protein (55). Interestingly, the fact that in ZR75.1 cells exposure to low doses of BPDE (50–100 nM) tended to increase BRCA-1 promoter activity may imply the existence of dose-dependent mechanisms that activate BRCA-1 expression independent of p53.

Because it is known that levels of BRCA-1 vary during the cell cycle, with minimal expression in G_0-G_1 (56), one could argue that the decrease in BRCA-1 mRNA in response to B[*a*]P/BPDE was an indirect consequence of cell cycle arrest. However, we consider this possibility unlikely because we observed in a previous study (23) and in the present (data not shown) study that both B[*a*]P and BPDE induced accumulation of MCF-7 cells in S-phase, at which interval expression of BRCA-1 was expected to peak (56). This interpretation is in accord with data, from other groups, documenting the arrest of MCF-7 cells in S-phase after exposure to benzo[*g*]chrysene-dihydrodiol epoxides (46). In addition, we considered the possibility that by forming DNA adducts BPDE might impede progression of RNA polymerase II on the transcribed strand. However, evidence that transient expression of MCF-7 cells with mutated p53 restored BRCA-1 transcription and protein levels suggested that p53 mediated the negative effects of BPDE on BRCA-1 transcription. Furthermore, the distinct mRNA expression profiles presented in Fig. 1 and those obtained by cDNA microarray analysis of >1000 CancerArray genes (data not shown) confirmed that the loss of BRCA-1 mRNA in cells treated with B[*a*]P or BPDE was not associated with general disruption of the transcriptional machinery.

Loss of BRCA-1 in cells harboring DNA damage may destine cells to lethality (57–60). Previous reports from our laboratory (22) indicated that in HBL-100 cells expressing the SV40 large T antigen, which is known to inhibit the transcriptional transactivation functions of p53 (54), neither BRCA-1 expression nor proliferation was affected by B[*a*]P. Conversely, in addition to reducing BRCA-1 expression, acute exposure to B[*a*]P induced cell death of 70–80% of breast MCF-7 cells, which express wild-type p53 (21). The accumulation of Bax-α mRNA as well as p53 and p21 protein was paralleled in this study by loss of Bcl-2 mRNA, suggesting that proapoptotic pathways were alerted in response to exposure to B[*a*]P. However, because of deficient expression of caspase-3, a key player in the signaling of programmed cell death, MCF-7 cells may not succumb through classic apoptosis (57). A significant scenario emerging from these observations is one in which cells resistant to the cellular stresses induced by PAHs (61), but with a reduced potential for BRCA-1 expression, may be more likely to undergo neoplastic transformation (62).

In summary, the present study provides novel insights into the mechanisms through which PAHs may adversely affect transcriptional activity of the *BRCA-1* gene. Activation of the AhR appears to be insufficient for repression of basal BRCA-1 transcription, which however, may be hampered after the bioactivation of AhR ligands to reactive metabolites, such as BPDE, and the gaining of p53 functions. The significance of these findings is that they offer a molecular basis for investigating the contribution of PAHs and structurally related compounds to dysregulation of the *BRCA-1* gene as well as their role as a risk factor in the etiology of sporadic breast cancer.

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Epigenetics of Breast Cancer: Polycyclic Aromatic Hydrocarbons as Risk Factors

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In the absence of a causal relationship between the incidence of sporadic breast cancer and occurrence of mutations in breast cancer susceptibility genes, efforts directed to investigating the contribution of environmental xenobiotics in the etiology of sporadic mammary neoplasia are warranted. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants, which have been shown to induce DNA damage and disrupt cell cycle progression. In this report we discuss published data pointing to PAHs as a risk factor in carcinogenesis, and present findings generated in our laboratory suggesting that the mammary tumorigenicity of PAHs may be attributable, at least in part, to disruption of BRCA-1 expression by reactive PAH-metabolites. We report that benzo[a]pyrene (B[a]P), selected as a prototype PAH, disrupts BRCA-1 transcription in estrogen receptor (ER)-positive but not ER-negative breast cancer cells. The reduced potential for BRCA-1 expression in B[a]P-treated cells coincides with disruption of cell cycle kinetics and accumulation of p53. These effects are counteracted by the AhR-antagonist α-naphthoflavone (ANF), and in breast cancer cells expressing mutant p53 or the E6 human papilloma virus protein. We suggest that exposure to PAHs may be a predisposing factor in the etiology of sporadic breast cancer by disrupting the expression of BRCA-1. Environ. Mol. Mutagen. 39:235–244, 2002. © 2002 Wiley-Liss, Inc.

Key words:1 polycyclic aromatic hydrocarbons; estrogen receptor; aromatic hydrocarbon receptor; p53; BRCA-1

INTRODUCTION

Breast cancer ranks second among cancer deaths in women. An estimated 182,800 new invasive cases of breast cancer and 40,800 deaths were estimated to occur among women in the United States during 2000. Some of the risk factors that contribute to the development of breast cancer include age, familial history, menstrual periods that started early in life, nulliparity, alcohol consumption, recent use of oral contraceptives or postmenopausal estrogens, and higher education and socioeconomic status [ACS, 2000]. One of the puzzles in breast carcinogenesis is that about 90–95% of breast cancers are sporadic and occur in women in the absence of mutations in breast cancer susceptibility genes including *BRCA-1* and *BRCA-2* [Peto et al., 1999; Shih et al., 2000]. In the absence of a causal relationship between the occurrence of sporadic breast cancer and mutations in breast cancer susceptibility genes, efforts directed to investigating the contribution of environmental xenobiotics are warranted. The objective of this report is to review published observations highlighting the role of endogenous factors as mediators of PAH/gene interactions in the etiology of breast cancer and present new data from our laboratory implicating PAHs as epigenetic effectors of BRCA-1 (Fig. 1).

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Abbreviations: ANF, α-naphthoflavone; AhR, aromatic hydrocarbon receptor; B[a]P, benzo[a]pyrene; B[e]P, benzo[e]pyrene; BPDE, 7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; ER, estrogen receptor; 3-MC, 3-methylcholanthrene; PAHs, polycyclic aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive elements.

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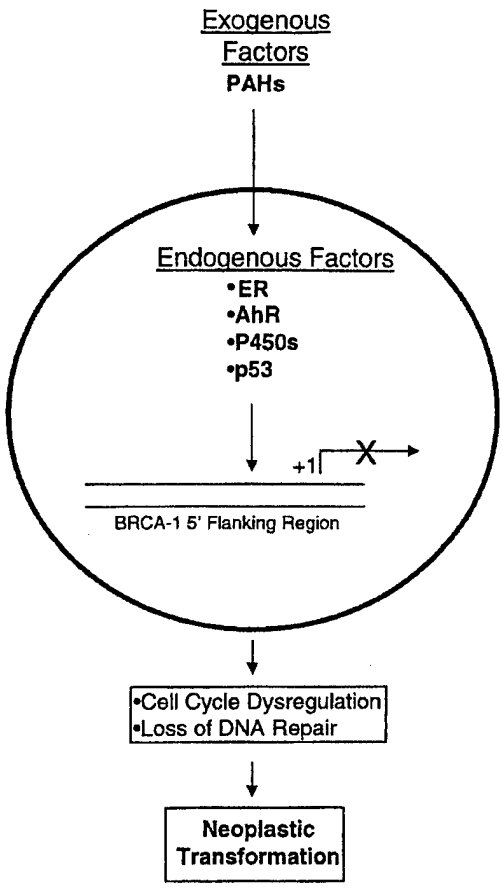

Fig. 1. PAHs as potential risk factors in breast carcinogenesis. Exposure to PAHs may target the expression of regulatory proteins involved in detoxification and DNA repair, thus altering the ability of cells to maintain genomic integrity. The susceptibility to PAHs may be influenced by the cellular complement of endogenous factors including the ER and AhR receptor, enzymes of the P450 family, and p53. These factors can modulate the expression of responsive genes (*BRCA-1*). Possible outcomes may be loss of expression of *BRCA-1* and DNA repair capacity, dysregulation of cell cycle control, and neoplastic transformation.

MATERIALS AND METHODS

Cell Culture and Chemicals

Breast (MCF-7, ZR75.1, HBL-100, MDA-MB-231) and ovarian (BG-1) cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Sigma Chemical, St. Louis, MO) containing phenol-red and supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) as described previously [Romagnolo et al., 1998]. For experiments involving estrogen, MCF-7 cells were preconditioned and cultured in 5% charcoal-stripped-FCS phenol red-free medium [Jeffy et al., 1999]. α-Naphthoflavone (ANF), anabasine (ANB), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), colchicine, 3-methylcholanthrene (3-MC), and nicotine (NCT) were obtained from Sigma. Aphidicolin was obtained from Calbiochem (La Jolla, CA). 7r,8t-Dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were obtained from Midwest Research Institute (Kansas City, MO).

Semiquantitative RT-PCR

For mRNA studies, cells were plated at a density of 2×10^6 cells/100-mm tissue culture dish and maintained in DMEM/F12 plus 10% FCS. Three dishes were assigned to each experimental treatment. Details concerning the experimental conditions for semiquantitative reverse transcriptase (RT)/polymerase chain reaction (PCR) analysis of BRCA-1 are described elsewhere [Jeffy et al., 1999]. Briefly, total RNA (400 ng) was incubated with random hexamer primers, Moloney murine leukemia virus-RT, RNase inhibitor (Life Technologies/Gibco BRL, Gaithersburg, MD), and RT buffer (Ambion, Austin, TX) at 42°C for 1 hr. The amplification products were of the expected size and their authenticity to the GenBank was confirmed by direct sequencing. Preliminary control experiments (data not shown) were carried out to ensure RT-PCR conditions allowed for linear amplification of PCR products. The expression levels of BRCA-1 were quantified by Alpha Imager (Alpha Innotech, San Diego, CA) analysis and corrected for the expression of the control mRNA (BRCA-1/S15).

Western Blotting

Western blotting was performed as described previously [Jeffy et al., 2000]. Cell extracts were normalized to protein content and separated by 4–12% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblotting was carried out with antibodies raised against BRCA-1 (Ab-2) and p53 (Ab-2) obtained from Oncogene Research Products (Cambridge, MA). Normalization of Western blots was confirmed by incubating immunoblots with β-actin antibody-1 (Oncogene Research Products). The immunocomplexes were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

BRCA-1 Promoter Studies

Details concerning isolation and cloning of a 1.69-kb BRCA-1 promoter fragment upstream of a luciferase reporter cassette (pGL3-BRCA-1) are described elsewhere [Jeffy et al., 2002]. For expression studies, the pGL3-BRCA-1 vector was transfected into breast cancer cells using the Lipofectamine-Plus procedure, as described by the manufacturer (Life Technologies). Variations in transfection efficiency were accounted for by cotransfection with plasmids encoding for renilla. Internal standards for luciferase activity were the pGL3Control vector containing an SV40 promoter, and pGL3Basic (Promega, Madison, WI) (data not shown). To control for the efficacy of treatments with B[a]P and TCDD, cells were transfected with plasmid p1A1–4x-Luc containing a CYP1A1 consensus sequence linked to an array of four GCGTG elements. Cell extracts were collected at 24 hr after treatment with B[a]P, TCDD, or BPDE. Luciferase reporter activity was monitored using a Luminometer 20/20 and expressed as relative luciferase units (Luc) corrected for renilla (Luc/renilla).

Flow Cytometry

Flow cytometry was performed as previously described [Andres et al., 1998]. Briefly, cells were harvested with trypsin and washed in phosphate buffer solution (PBS). Then cells were treated with RNAse and stained with propidium iodide (50 μg/ml in PBS). Cell cycle distribution profiles were recorded with a FACscan (Becton-Dickinson Immunocytometry Systems, Braintree, MA), using a CELLQuest program. Data were analyzed with the MODFIT.2 software at the Flow Cytometry Laboratory of the Arizona Cancer Center.

Statistical Analysis

For transfection and flow cytometry experiments, data were presented as means ± standard error. Statview statistical analysis software was used for

analysis of variance and multiple comparisons by Fisher's protected least-significant difference test.

RESULTS AND DISCUSSION

PAHs as Exogenous Risk Factors in Carcinogenesis

Cigarette smoke, diet, and environmental pollution contain a complex mixture of compounds including PAHs, aromatic amines, and nitrosamines, all of which after metabolic activation may induce DNA damage [Szeliga et al., 1997; Dipple et al., 1999]. In the absence of efficient DNA repair, the resulting aberrations may initiate carcinogenesis [Wei et al., 1996; Minamoto et al., 1999]. Of the many substances present in tobacco smoke, B[a]P is considered a prototype PAH, and classic DNA damaging agent and carcinogen. B[a]P is a ubiquitous pollutant found in amounts of 10 ng per cigarette contributing about 200 ng/day for a pack-a-day smoker [Scherer et al., 2000]. Food ingestion contributes to significant exposure to B[a]P. Charcoal-broiled steaks and ground meat had B[a]P concentrations up to 50 ng/g of meat [Lijinsky, 1991]. Recent epidemiological studies concerning foods in diets of subjects residing in the Washington, DC metropolitan area revealed that the highest levels of B[a]P were found in cooked meat (4 ng B[a]P/g). In contrast, nonmeat items contained up to 0.5 ng/g [Kazerouni et al., 2001]. The daily dietary intake of B[a]P has been estimated to range from 120 to 2800 ng/day [Hattemer-Frey et al., 1991], with average values approximating 600 ng/day [Scherer et al., 2000]. Catabolism of PAHs can generate reactive diol-epoxides, which have been shown to form stable DNA adducts at mutational hotspots in the *p53* and Harvey-*ras* genes, disrupt transcription, and the binding affinity of Sp1 and E2F transcription heterodimers to DNA [MacLeod et al., 1995; Denissenko et al., 1996; Butler et al., 1997].

Defective repair of PAH:DNA adducts have been related to transversion mutations in the *p53* tumor-suppressor gene and the incidence of various types of cancer including lung cancer, head and neck cancers, and esophageal carcinomas [Denissenko et al., 1996]. These observations support the notion that incapacitated DNA repair leads to accumulation of PAH-induced chromosomal breakage. The profile of DNA mutations is influenced by the intensity and duration of exposure to metabolites of PAHs. For example, the intracellular concentration of the B[a]P-diol epoxide BPDE may influence the balance between cytotoxicity and mutagenicity [Quan et al., 1995]. An increased proportion of mutations at the A:T base pairs relative to the G:C base pairs was observed for low doses (40 nM) compared to that for higher doses (500 nM) of BPDE, suggesting dose-dependent differences in the profile of mutations [Wei et al., 1991]. Removal of many types of DNA damage requires transcription-coupled repair, which is highly efficient in transcriptionally active DNA where lesions in the transcribed strand are rapidly repaired [Gowen et al., 1998]. Therefore, it is conceivable that dose-dependent downregulation by PAHs of DNA-repair genes may compromise genome integrity. A possible consequence may be the accumulation of mutations in genes involved in cell cycle regulation and the development of neoplastic transformation.

TABLE I. Binding Affinity of Various PAHs for the AhR[a]

Selected PAH	EC-50 receptor binding values
High affinity	
TCDD	1.0×10^{-8} M
3-Methylcholanthrene	2.8×10^{-8} M
Intermediate affinity	
7,12-Dimethylbenzo[a]anthracene	3.2×10^{-7} M
Benzo[a]pyrene	3.6×10^{-7} M
11-Methylbenzo[a]anthracene	1.3×10^{-6} M
Low affinity	
2,3-Benzofluorene	1.3×10^{-5} M
Benzo[e]pyrene	1.0×10^{-4} M

[a]Data from the Piskorska-Pliszczynska et al. [1986].

Endogenous Factors of Susceptibility to PAHs: AhR and ER Pathways

At the cellular level, PAHs activate multiple and overlapping signal transduction pathways, which must be regarded as an integral part of a cellular network [Shakelford et al., 1999]. PAHs can induce tumor initiation and promotion through several, likely combinatorial, mechanisms including negative effects on transcription of tumor-suppressor genes [Denissenko et al., 1996], transcriptional activation of protooncogenes [Bral et al., 1996], and activation of enzymes that oxidize procarcinogenic PAHs to reactive metabolites [Aust et al., 1981]. In mammalian models, responsiveness to PAHs is mediated, among other factors, by the aromatic hydrocarbon receptor (AhR) pathway. PAHs and polyhalogenated hydrocarbons, including B[a]P, B[e]P, 3-MC, and TCDD display various binding affinities for the AhR (Table I).

The AhR is a ligand-activated transcription factor that influences the transcription of several genes and mediates the induction of DNA damage, growth arrest, and apoptosis in various cell systems [Nebert et al., 1990]. In the absence of exogenous ligands the AhR shuttles between the nucleus and the cytosol. However, binding of ligands elicits an increase in the rate of nuclear import of the AhR [Richter et al., 2001]. Activation of the AhR signal transduction pathway necessitates translocation to the nucleus of the liganded AhR and its subsequent association with nuclear translocator and other accessory proteins to form a heterocomplex [Henry et al., 1993; Hord et al., 1994]. This heterocomplex influences the expression of responsive genes through interactions with xenobiotic responsive elements (XRE =

5′-GCGTG-3′) in promoter regions, thus inducing alterations in the chromatin structure, which facilitate access to transcription factors [Safe, 2001]. Several structurally diverse compounds can antagonize the activation of the AhR pathway. One such antagonist is α-naphthoflavone (ANF), which competes for the cytosolic AhR receptor binding sites, thus eliciting a conformational change that lowers the affinity for DNA [Gasiewicz and Rucci, 1991]. As a part of this inhibition, ANF prevents the activation of the B[a]P-hydroxylase and exerts protective effects against carcinogens [Merchant et al., 1990].

PAHs exert a number of pleiotropic responses including mammary tumors in rodents [Ronai et al.,1994], dermal toxicity, immune reactivity, and disruption of endocrine functions [Krishnan et al., 1995; Hoivik et al., 1997]. Many investigations have dealt with cross talk between the AhR and ER pathways and disruption of estrogen responses by PAHs and AhR-ligands in breast cancer cells [Krishnan et al., 1995; Safe and Krishnan, 1995]. Indeed, understanding how activation of the AhR-mediated pathway prevents estrogen-stimulated growth, thus halting progression through the cell cycle, has been central to the development of selective AhR modulators for treatment of breast cancer [Safe et al., 1999]. Whereas the susceptibility to PAHs has been attributed to the concomitant expression of the ER and AhR [Safe and Krishnan, 1995], other studies have suggested [Dohr et al., 1995] that modulation of expression of PAH-sensitive genes may depend on transcription factors or receptors that bind to the ER [Spink et al., 1998].

The *BRCA-1* Gene as a Molecular Target of Exposure to PAHs

The *BRCA-1* gene encodes for a phosphoprotein of 1863 amino acids [Miki et al., 1994]. Mutations in *BRCA-1* predispose to the development of primary breast and ovarian carcinomas [Futreal et al., 1994]. Although the function of *BRCA-1* is still under investigation, several lines of evidence indicate that the BRCA-1 protein localizes to the nucleus and participates directly or indirectly in transcription-coupled repair of oxidative damage to DNA. Association of the BRCA-1 protein with the RNA polymerase II holoenzyme, which is necessary for transcription from DNA, suggests that *BRCA-1* participates in the regulation of transcription [Gowen et al, 1998].

In mitotic and meiotic cells, both BRCA-1 and BRCA-2 interact with rad51 [Scully et al., 1997; Sharan et al., 1997]. The rad51 protein is involved in repairing double-stranded breaks and recombination-linked repair offering one paradigm that both BRCA-1 and BRCA-2 may be involved in maintaining the integrity of the genome [Kinzler and Vogelstein, 1997; Andres et al., 1998]. Normal expression of BRCA-1 is associated with cell growth retardation and tumor inhibition [Holt et al., 1996; Somasundaram et al., 1997], whereas depressed expression of BRCA-1 contributes to accelerated growth [Thompson et al., 1995; Rao et al., 1996; Larson et al., 1997].

The BRCA-1 protein is expressed in a cell-cycle-dependent manner [Chen et al., 1996] and peaks at the G1/S boundary [Rajan et al., 1996]. One mechanism by which BRCA-1 may induce cell cycle arrest is through interactions with a variety of molecules including the tumor suppressors *p53*, *p21*, and pRb [Deng and Brodie, 2000]. Regulation of phosphorylation of BRCA-1 during the cell cycle or in response to DNA damage may contribute to the function of BRCA-1 [Thomas et al., 1997]. Not only is BRCA-1 hyperphosphorylated after exposure to DNA-damaging agents, but also its pattern of subcellular distribution changes. These data advocate a potential involvement of BRCA-1 in cell cycle checkpoint or repair of DNA damage.

Disruption of *BRCA-1* Expression by AhR-ligands

In detailing the function of *BRCA-1* as a tumor-suppressor gene, it is imperative to assess the relationships between regulation of cell proliferation and *BRCA-1* expression. Growth stimulation of ER-positive (MCF-7) breast cancer cells with estrogen correlated with increased expression of *BRCA-1* [Gudas et al., 1995; Marks et al., 1997]. Conversely, expression of *BRCA-1* in breast ER-negative (HBL-100 and MDA-MD-231) cancer cells was not influenced by estrogen, suggesting that ER status may be important for regulation of *BRCA-1*. The antiestrogen ICI-182780 inhibited estrogen-induced cell proliferation and *BRCA-1* expression in ER-positive cells, thus confirming the participation of the ER pathway [Romagnolo et al., 1998].

Based on the information that PAHs can disrupt the expression of estrogen-inducible genes [Safe et al., 1999], we investigated the effects of acute and chronic exposure to B[a]P on *BRCA-1* expression in ER-positive breast MCF-7 and ovarian (BG-1) cancer cells. Basal *BRCA-1* mRNA and protein levels were reduced both after chronic (40 nM) or acute (0.5 to 5 μM) exposure to B[a]P [Jeffy et al., 1999]. Moreover, B[a]P abrogated estrogen-induced expression of *BRCA-1* in a dose- and time-dependent fashion. Loss of *BRCA-1* was accompanied by a dose-dependent increase in cell death [Jeffy et al., 2000]. These data suggested that the effects of B[a]P on *BRCA-1* expression and cell viability were influenced by the interaction between intensity and duration of exposure. Acute treatment of ER-negative MDA-MB-231 and HBL-100 cells with B[a]P did not affect cell proliferation and *BRCA-1* expression levels [Jeffy et al., 1999]. The fact that ER-negative cells were refractory to B[a]P could not be explained by the lack of a functional AhR pathway. In agreement with other investigations [Spink et al., 1998], we detected constitutive expression of the AhR and various degrees of induction of *CYP1A1* mRNA by B[a]P in all of breast cancer cell lines used in this study irrespective of ER status. Thus, upregulation of *CYP1A1* by B[a]P correlated with reduced *BRCA-1* only in

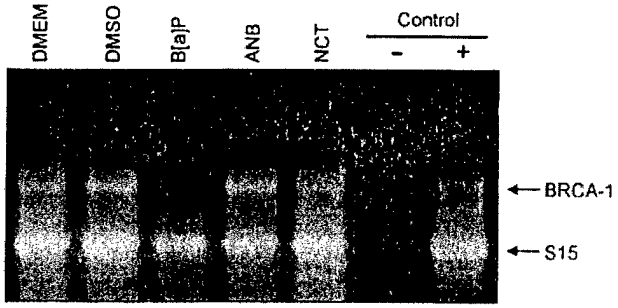

Fig. 2. Effects of tobacco derivatives on *BRCA-1* mRNA levels. Total RNA was obtained from MCF-7 cells cultured for 72 hr in control medium (DMEM), or DMEM plus vehicle (DMSO), 5 μM B[a]P, and 200 μM ANB or NCT. Controls were (−) no total RNA in RT-PCR reactions or (+) RNA from mouse liver (Ambion). Bands represent *BRCA-1* (712 bp) mRNA spanning exon-1A to exon-8 and S15 (488 bp) ribosomal subunit (Ambion), which was used as an internal control for PCR conditions and equal loading. Relative densitometric ratios (BRCA-1/S15 mRNA) were: DMEM = 0.50, DMSO = 0.52, B[a]P = 0.1, ANB = 0.60, NCT = 0.52. Compared to DMEM, treatment with B[a]P reduced *BRCA-1* mRNA levels by 5.0-fold. Conditions for quantitative RT-PCR analysis of *BRCA-1* mRNA are described elsewhere [Jeffy et al., 1999].

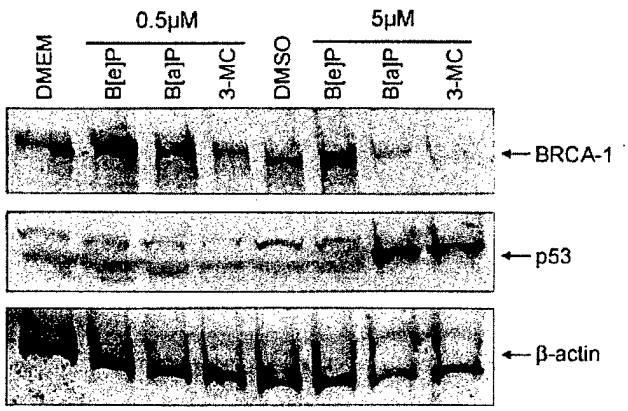

Fig. 3. Effects of AhR-ligands on BRCA-1 and p53 protein levels. MCF-7 cells were cultured in control medium (DMEM), or DMEM plus vehicle (DMSO), 0.5 and 5.0 μM B[e]P, B[a]P, and 3-MC. Cell lysates were tested for levels of BRCA-1 and p53 by Western blot analysis as described previously [Jeffy et al., 2000]. The control bands are β-actin immunocomplexes. BRCA-1 protein levels were reduced in the order 3-MC > B[a]P > B[e]P.

ER-positive breast MCF-7 and ovarian BG-1 cancer cells. In contrast, neither *BRCA-1* nor cell viability (data not shown) was influenced by treatment of MCF-7 cells with other tobacco derivatives including anabasine (ANB) and nicotine (NCT) (Fig. 2).

When we compared the effects of B[e]P, B[a]P, and 3-MC, we observed that the ability of these AhR-ligands to inhibit BRCA-1 protein levels (3-MC > B[a]P > B[e]P) (Fig. 3) directly correlated to their binding affinity for the AhR (Table I). Moreover, loss of BRCA-1 induced by B[a]P and 3-MC correlated with upregulation of p53. Supporting evidence for the involvement of the AhR pathway in the B[a]P-mediated repression of BRCA-1 was provided by competition experiments with the AhR antagonist ANF, which antagonizes PAHs by forming an inactive complex with the AhR [Gasiewicz and Rucci, 1991]. At concentrations ranging from 10 to 25 μM, ANF hampered the negative effects of B[a]P on cell viability while restoring BRCA-1 and p53 protein to control levels [Jeffy et al., 2000]. These findings confirmed the involvement of the AhR in the inhibition of *BRCA-1* expression induced by B[a]P, and corroborated the existence of an inverse relationship between BRCA-1 and p53 levels in breast cancer cells [MacLachlan et al., 2000].

Repression of *BRCA-1* Transcription by the Metabolite BPDE Requires Functional p53

In searching for a mechanism responsible for PAH-mediated repression of *BRCA-1,* we examined the effects of the AhR-ligand TCDD, which displays high affinity for the AhR (Table I) but it is not metabolized. The treatment of MCF-7 cells with TCDD had no effects on basal *BRCA-1* mRNA and protein levels [Jeffy et al., 1999]. This was in spite of the fact that TCDD greatly enhanced the expression of the *CYP1A1* gene and hampered cell viability in a dose-dependent fashion (10 to 500 nM) (data not shown). Our interpretation of these observations is, first, that loss of *BRCA-1* expression was not a direct effect of reduced cell viability and, second, that activation of the AhR was not sufficient to repress *BRCA-1* expression. This contention was tested further by transfecting MCF-7 cells with a 1.7-kb-BRCA-1 promoter-luciferase expression vector (pGL3BRCA-1). These experiments confirmed that B[a]P (Fig. 4A), but not TCDD (data not shown), repressed basal transcription of the *BRCA-1* reporter construct, although both AhR-ligands induced the activity of a promoter segment containing an array of four XRE (p1A1–4x-LUC) [Jeffy et al., 2002].

These cumulative data suggested that the activated AhR was likely not responsible for the B[a]P-dependent repression of basal *BRCA-1* transcription. Alternatively, we speculated that metabolites of B[a]P may repress *BRCA-1* promoter activity or alter the expression of other factors important in *BRCA-1* regulation. This inference was supported by our earlier observation that the B[a]P-metabolite BPDE reduced the potential for *BRCA-1* expression [Jeffy et al., 1999]. The exposure of MCF-7 cells to the metabolite BPDE exerted a long-lasting repressive effect on *BRCA-1* mRNA and protein levels, given that pretreatment with BPDE (1–6 hr) reduced the potential for *BRCA-1* expression at later time points (72 hr) [Jeffy et al., 2000]. In keeping with these findings, treatment of MCF-7 cells with BPDE (500 nM) reduced by 1.9-fold *BRCA-1* transcription (Fig. 4B) and 5.0-fold BRCA-1 protein [Jeffy et al., 2002].

The exposure to PAHs has been shown to contribute to

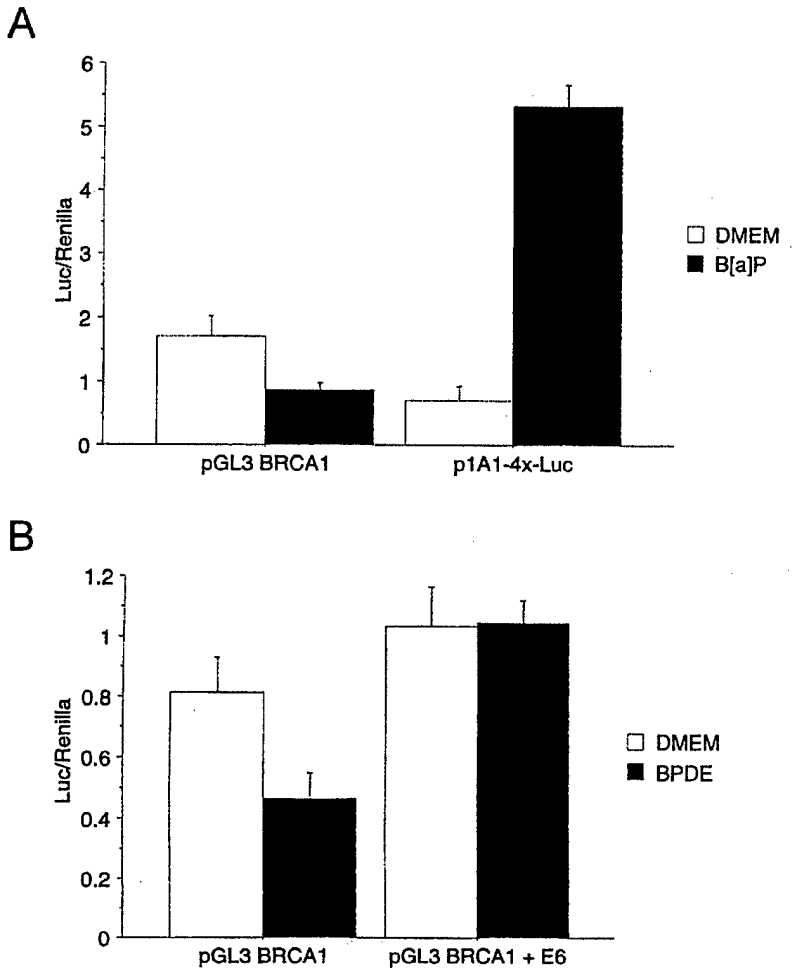

Fig. 4. Repression of BRCA-1 transcription by B[a]P and BPDE. MCF-7 cells were transfected with pGL3BRCA-1 or p1A1–4x-Luc. **A:** Treatment with B[a]P (5 μM) for 48 hr repressed (2.0-fold, $P < 0.01$) luciferase (Luc) reporter activity in cells transfected with pGL3BRCA-1. Reporter activity of the positive control promoter (1A1-4x-Luc) containing an array of four XRE = GCGTG was stimulated 9.0-fold ($P < 0.01$) by B[a]P compared with control medium (DMEM). **B:** Treatment of MCF-7 cells for 24 hr with 500 nM BPDE reduced (1.9-fold, $P < 0.01$) Luc/renilla activity. In contrast, cotransfection with an expression vector encoding for the E6 human papilloma virus counteracted the negative effects of BPDE on BRCA-1 promoter activity. The E6 gene product binds to p53 and leads to suppression of its biological functions. These data suggested that p53 mediated the negative effects of the metabolite BPDE on BRCA-1 promoter activity [Jeffy et al., 2002].

DNA damage, cell growth arrest [Khan et al., 1997], and accumulation of p53 [Khan et al., 1998]. In our studies, treatment of MCF-7 cells with B[a]P induced a significant increase in p53, which was stabilized at levels significantly higher than those observed in control cells [Jeffy et al., 2000]. The accumulation of p53 in B[a]P-treated cells was followed by an increase in the levels of cyclin-dependent kinase inhibitor p21. In contrast, there were no apparent changes in the content of p27, whereas the cellular levels of the p53-regulator mdm2 increased after treatment with B[a]P. When comparing the temporal profiles of expression of BRCA-1 and p53, the accumulation of p53 in B[a]P-treated cells preceded the loss of BRCA-1 [Jeffy et al., 2000].

Based on the observation that *BRCA-1* may be negatively regulated by *p53* [MacLachlan et al., 2000], we tested whether inhibition of *BRCA-1* transcription by B[a]P and BPDE resulted from gain of *p53* functions. In keeping with this hypothesis, transfection of MCF-7 cells, which contain wild-type *p53* [Greenblatt et al., 1994], with a vector encoding *p53* mutated at amino acid position 175 (R to H) counteracted the negative effects of B[a]P and BPDE on *BRCA-1* promoter activity [Jeffy et al., 2002]. Our interpretation of these findings was that transient expression of mutant *p53* interfered with normal *p53* functions in a trans-dominant negative fashion. Similarly, transient transfection of MCF-7 cells with an expression vector encoding the E6 human papilloma virus counteracted the negative effects of BPDE on *BRCA-1* promoter activity (Fig. 4B). The E6 gene product binds to *p53* and leads to suppression of its biological functions [Mietz et al., 1992]. From these cumu-

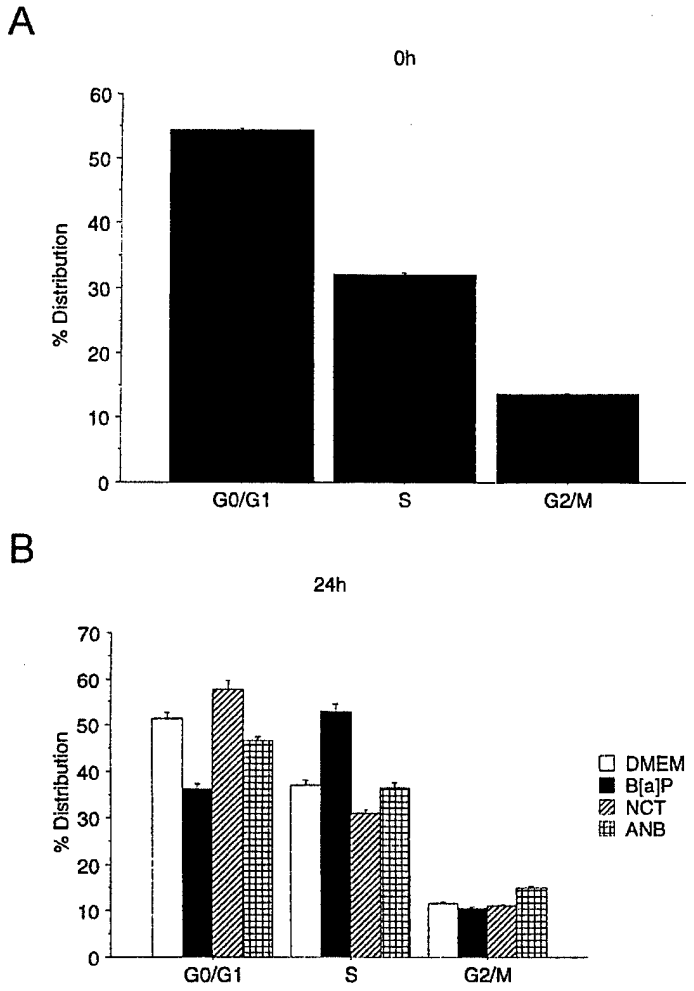

Fig. 5. B[a]P delays transit of MCF-7 cells through S phase. **A:** Asynchronous MCF-7 cells were cultured for 24 hr in control medium (DMEM) or (**B**) DMEM plus 5 μM B[a]P, and 200 μM anabasine (ANB) or nicotine (NCT). Cell cycle distribution was examined by flow cytometry after propidium-iodide staining as described under Materials and Methods. Bars represent means ± SE percentage distribution of MCF-7 cells before (0 hr) or after (24 hr) treatment from two independent experiments. Treatment with B[a]P increased by 1.5-fold ($P < 0.01$) the percentage of cells positioned in S phase compared with control DMEM.

lative data, we concluded that activation of the AhR pathway was required, but not sufficient for B[a]P-mediated inhibition of *BRCA-1* transcription. Rather, we envisioned that the metabolite BPDE elevated *p53,* which in turn, inhibited *BRCA-1* promoter activity. This contention is supported by the fact that repression of *BRCA-1* transcriptional activity by B[a]P or BPDE could not be seen in breast cancer ZR75.1 cells containing mutated *p53* (152 P to L) [Jeffy et al, 2002].

PAHs Disrupt Cell Cycle Checkpoints and Progression

Pausing in S phase and stabilization of *p53* have been observed in MCF-7 cells upon treatment with benzo-[g]chrysene dihydrodiol epoxide enantiomers [Khan et al, 1998]. The treatment of asynchronous MCF-7 cells (Fig. 5A) with B[a]P induced a significant enrichment of cells in S phase, which was paralleled by a reduction in the fraction of cells positioned in G0/G1 (Fig. 5B). Conversely, neither NCT nor ANB altered cell cycle distribution. Cotreatment with the AhR-antagonist ANF restored normal cell cycle distribution [Jeffy et al., 1999], and BRCA-1 and p53 protein levels [Jeffy et al., 2000]. These studies indicated that in ER-positive MCF-7 cells, repression of *BRCA-1* by B[a]P required the participation of the AhR pathway and corroborated the existence of an inverse relationship between BRCA-1 and p53 status [MacLachlan et al., 2000].

Because BRCA-1 protein normally peaks in S phase [Chen et al., 1996], we attempted to characterize the effects of B[a]P in MCF-7 cells synchronized with aphidicolin in S phase. Overall, B[a]P altered normal cell

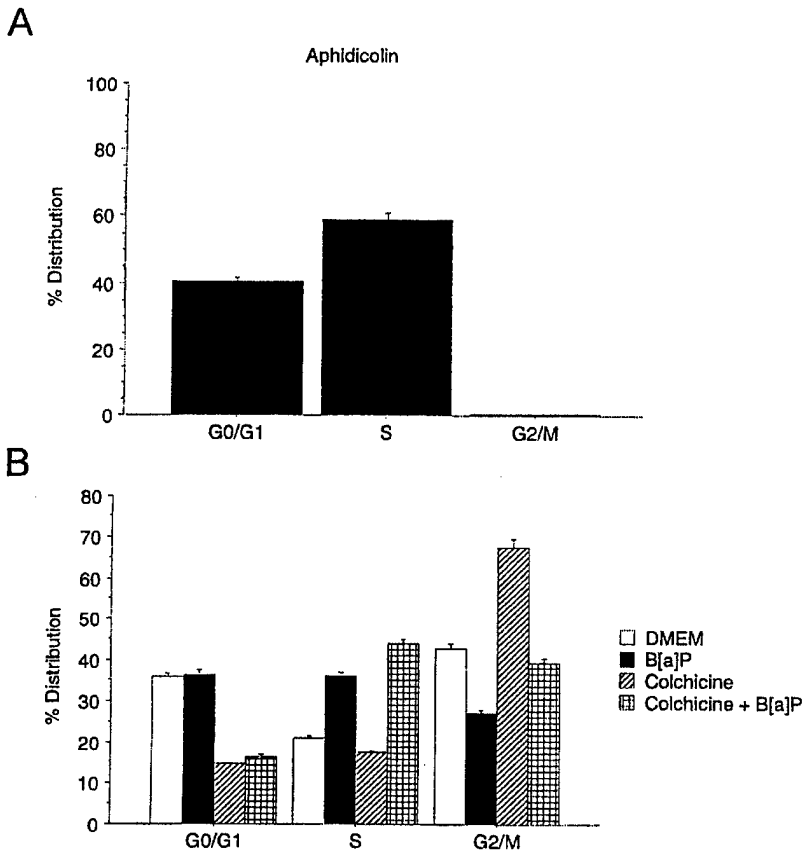

Fig. 6. G2/M-trapping experiments. **A:** MCF-7 cells were synchronized in S phase by treatment with aphidicolin for 24 hr. **B:** Cells were released into control DMEM medium or DMEM containing 5 μM B[a]P, 0.25 μM colchicine, or 5 μM B[a]P plus 0.25 μM colchicine. Cell cycle distribution was examined at 12 hr by flow cytometry after propidium-iodide staining as described under Materials and Methods. Bars represent means ± SE percentage distribution of MCF-7 cells from two independent experiments. The treatment with B[a]P increased the percentage of cells in S phase (1.8-fold, $P < 0.01$), thus delaying transit to G2/M [Jeffy et al., 2000].

cycling by lengthening the transition through S and inducing accumulation in G2/M [Jeffy et al., 2000]. These results were confirmed in G2/M trapping experiments, in which cells previously synchronized in S phase with aphidicolin (Fig. 6A) were released into culture media containing colchicine to prevent cycling beyond G2/M. These experiments documented that B[a]P increased the percentage of cells in S phase (1.8-fold, $P < 0.01$) (Fig. 6B). However, this checkpoint was relaxed because cells resumed cycling to G2/M by 24 hr with reduced BRCA-1 levels [Jeffy et al., 2000].

CONCLUSIONS

Taken together, these data suggest that B[a]P, a prototype PAH found in tobacco smoke, environmental pollution, and foods may contribute to breast carcinogenesis through the inhibition of the tumor-suppressor gene *BRCA-1*. This effect is not limited to B[a]P, given that other PAHs such as 3-MC repress BRCA-1 protein levels in breast cancer cells. In this report we highlighted the possibility that the carcinogenicity of PAHs may be a function of intensity and duration of exposure, binding affinity for the AhR, and ER and p53 status. At noncytotoxic concentrations (i.e., chronic exposure), PAHs may contribute to breast tumorigenesis through chronic inhibition of DNA repair functions requiring *BRCA-1*, thus favoring the fixation of mutations caused by reactive metabolites of PAHs. On the other hand, the exposure to cytotoxic concentrations of PAHs (i.e., acute exposure) may allow proliferation of resistant cells [Moore et al., 1994] with low *BRCA-1* but containing mutations or chromosomal aberrations. To date, no mutations in the *BRCA-1* gene have been identified in sporadic breast cancers, whereas the expression levels of *BRCA-1* in breast tumors are lower than those observed in normal mammary tissue [Thompson et al., 1995]. These studies offer preliminary evidence that bioactivation of AhR-ligands may be a risk factor in the etiology of sporadic breast cancer by lowering *BRCA-1* expression.

ACKNOWLEDGMENTS

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